THESIS / DISSERTATION SUBMISSION

Title: **Pilot-scale wastewater surveillance for pathogenic yeasts in Mangaung, South Africa**

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Contents

Motivation

Introduction

Fungal infections and their associated severity have gained increasing attention, yet we still understand very little about these microorganisms. The latest estimates on invasive fungal infections report an annual infection incidence of 6.5 million and 3.8 million deaths of which 2.5 million are directly attributed to fungal infections (Denning, 2024). Pathogenic yeast is an especially concerning group of fungi. These yeasts are often opportunistic pathogens that affect the health of immunocompromised or suppressed individuals, including patients with asthma, and cancer (Pour et al., 2018; Bongomin et al., 2017), and those receiving aggressive therapeutic approaches (Alcazar-Fuoli and Mellado, 2014). The total burden of fungal death is still unclear, for several reasons such as lack surveillance in some countries or surveillance of only selected fungal species (Denning, 2024).

The World Health Organisation (WHO) released a fungal priority pathogens list in 2022, classifying fungal species in critical, high and medium risk groups. This list caused most of the attention to shift towards the listed species, including pathogenic yeast from the *Candida* genus, which are found in all three groups. *C. auris* and *C. albicans* are found in the critical-risk group, *C. glabrata* (*Nakaseomyces glabrata*), *C. parapsilosis* and *C. tropicalis* in the high-risk group and lastly *C. krusei* (*Pichia kudriavzevii*) in the medium risk group. Cryptococcal species also make an appearance in the critical group (*Cryptococcus neoformans*) and medium-risk group (*Cryptococcus gattii*). These genera are found in our surrounding environments (Fisher et al., 2022) as well as part of our microbiome (*Candida* species) thus exposing us to the constant threat of infection if our immune systems were to be compromised. The severity of these pathogens will be discussed in the following chapters of this dissertation.

Although there are treatments available for fungal infections, we are faced with an additional challenge: antifungal resistance. This has become an increasing global concern due to its common occurrence (Berman and Krysan, 2020) and makes the effective treatment of fungal infections difficult, while there is already a limited number of available antifungal drugs (Sanglard 2016). Yeast can either be intrinsically resistant to certain antifungals or can develop acquired resistance due to prolonged exposure to the antifungal (Snelders et al., 2008) which induces genetic changes (Chaabane et al., 2019; Krishnasamy et al., 2018) in the yeast. Numerous studies have reported that resistance of certain

clinical isolates of fungal infections were of environmental origin (Chowdhary and Meis, 2018; Verweij et al., 2009), which suggests that antifungal-resistant strains from the environment (intrinsic or acquired) can infect immunocompromised or suppressed individuals.

Based on this knowledge, there is a clear need to fill the gaps in our knowledge and to continuously update research on these microorganisms.

Condition of wastewater treatment plants and transport systems in Bloemfontein, South Africa

Since the environment might be an important source of resistant strains and needs to be investigated, wastewater treatment plants (WWTP) are of particular interest. Wastewater treatment plants are a hub for pathogens shed by infected individuals or from agricultural run-off along with pharmaceuticals used to combat these pathogens. Antifungals are used in almost every industry (Li et al., 2019; Toda et al., 2019; Dalhoff 2017) making their presence in wastewater highly likely. So, antifungals and yeast are in close proximity to each other in wastewater, creating an environment favourable for resistance development. This leads us to speculate that treatment plants might be an important source of resistant yeast strains.

The following information is a summary of the Green Drop watch report for wastewater treatment plants in South Africa for the year 2023. The Department of Water and Sanitation SA uses this annual audit to assess the performance of WWTP in South Africa. This report determined that 44% of WWTP in the Free State are in critical condition, which can be defined as a wastewater system that receives a Green Drop (GD) score of less than 31%, meaning that most or all of the systems, processes and infrastructure responsible for delivery of safe wastewater services, have failed and or are dysfunctional.

The Free State was reported as the third highest critical state WWTPs in South Africa, following North West at 60% critical, and the Northen Cape at 59% of WWTP in a critical state. There are 96 WWTPs in the Free State as of 2021 with 64 in critical condition resulting in 67% of the Free State's WWTPs being in critical state. The top three Free State municipalities contributing to these scores include Mangaung, Matjhabeng and Maluti-A Phofung (23 of 64 systems). The GD Score for the Free State from 2013 to 2021 regressed from 51% to 26% while the provincial Risk Ratio for treatment plants regressed from 77% in 2013 to 81.2% in 2021. The most prominent risks were observed at a treatment level and pointed to WWTWs that exceeded their design capacity, dysfunctional processes and equipment

(especially disinfection), and effluent and sludge non-compliance with set health standards. Opportunities to improve on these conditions were presented by reducing cost through process optimisation, improved energy efficiency and beneficial use of sludge, nutrients, biogas, and other energy resources. However the Corrective Action Plans (CAP) Implementation Status: (Progress @ 31 March 2022) indicated no progress made for one of the Free State municipalities, this was after compliance letters were sent.

As can be seen from the information above and from Figure 1, WWTPs in the Mangaung metropole area often do not work due to poor maintenance or power disruptions, causing treatment plants and surrounding environments to be flooded by wastewater. This can pose a serious health risk to humans and animals who come into contact with this water. In addition, animals drink and tread through this water transferring potential pathogens to new environments. Sewage transport systems can also exceed their design capacity causing pipes to burst and wastewater to spill into the streets of residential or industrial areas (Figure 1). This increases the pool of individuals who are at risk of coming into contact with a variety of pathogens, including yeasts. As stated above, these yeast may not just be pathogenic but also resistant to some or all antifungals.

Figure 1 Poor condition of wastewater transport systems and treatment plants in Bloemfontein, Mangaung metropole, South Africa. A) wastewater spilling into streets and other residential areas. B) flooded treatment plant and cattle drinking and treating through wastewater which can then be transferred to new environments and or affect the health of humans and animals

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Considering the information presented in this motivation, it is more evident that research into the relationship between wastewater, antifungals and yeast is needed to understand how these components might interact and influence each other, and ultimately affect the health of the

community. One system that might be useful in this endeavour is wastewater surveillance which is not a new concept (Hart and Halden 2020; Paul et al., 1939) but has yet to be optimised for pathogenic yeast surveillance, thus, motivating this study.

References

Akinbobola AB, Kean R, Hanifi SMA, Quilliam RS (2023) Environmental reservoirs of the drug-resistant pathogenic yeast *Candida auris*. PLoS Pathog 13: 19:e1011268. doi: 10.1371/journal.ppat.1011268

Alcazar-Fuoli L, Mellado E (2014) Current status of antifungal resistance and its impact on clinical practice. Br J Haematol 166: 471-484. doi: https://doi.org/10.1111/bjh.12896

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases – estimate precision. J Fungi 3: 57. doi: https://doi.org/10.3390/jof3040057

Berman J, Krysan DJ (2020) Drug resistance and tolerance in fungi. Nat Rev Microbiol 18: 319–331. doi: https://doi.org/10.1038/s41579-019-0322-2

Chaabane F, Graf A, Jequier L, Coste AT (2019) Review on antifungal resistance mechanisms in the emerging pathogen *Candida auris*. Front Microbiolol 10: 2788. doi: https://doi.org/10.3389/fmicb.2019.02788

Chowdhary A, Meis JF (2018) Emergence of azole resistant *Aspergillus fumigatus* and one health: time to implement environmental stewardship. Environ Microbiol 20: 1299–1301. doi: 10.1111/1462-2920.14055

Dalhoff A (2017) For debate: May the use of the polyene macrolide natamycin as a food additive foster emergence of polyene-resistance in *Candida* species? Clin Microbiol 6: 4. doi: https://doi.org/10.4172/2327-5073.1000287

Denning DW (2024) Global incidence and mortality of severe fungal disease. Lancet infect dis 24: e428-38. doi: https://doi.org/10.1016/ S1473-3099(23)00692-8

Fisher MC, Alastruey-Izquierdo A, Berman J et al. (2022) Tackling the emerging threat of antifungal resistance to human health. Nat Rev Microbiol 20: 557–571. doi: https://doi.org/10.1038/s41579-022-00720-1

Green drop watch report (2023) Department of water and sanitation, republic of South Africa.

Hart OE, Halden RU (2020) Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy, opportunities and challenges. Sci Total Environ 730: 138875. doi: https://doi.org/10.1016/j.scitotenv.2020.138875

Krishnasamy L, Krishnakumar S, Kumaramanickavel G, Saikumar C (2018) Molecular mechanisms of antifungal drug resistance in *Candida* species. J Clin Diag Res 12: DE01-DE06. doi: 10.7860/JCDR/2018/36218.11961

Li S, Wu Q, Sun Q, Coffin S, Gui W, Zhu G (2019) Parental exposure to tebuconazole causes thyroid endocrine disruption in zebrafish and developmental toxicity in offspring. Aquat Toxicol 211: 116-123. doi: https://doi.org/10.1016/j.aquatox.2019.04.002

Paul JR, Trask JD, Culotta CS (1939) Poliomyelitic virus in sewage. Science 90: 258-259.

Pour AH, Salari S, Almani PGN (2018) Oropharyngeal candidiasis in HIV/AIDS patients and non-HIV subjects in the Southeast of Iran. Curr Med Mycol 4: 1-6. doi: https://doi.org/10.18502/cmm.4.4.379

Sanglard D (2016) Emerging threats in antifungal-resistant fungal pathogens. Front Med 3: 11. doi: https://doi.org/10.3389/fmed.2016.00011

Snelders E, van der Lee HAL, Kuijpers J, Rijs AJMM, Varga J, Samson RA, Mellado E, Donders ART, Melchers WJG, Verweij PE (2008) Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med 5: e219. doi: 10.1371/journal.pmed.0050219

Toda M, Beer KD, Kuivila KM, Chiller TM, Jackson BR (2019) Trends in Agricultural Triazole Fungicide Use in the United States, 1992–2016 and Possible Implications for Antifungal-Resistant Fungi in Human Disease. Environ Health Perspect 129: CID: 055001. doi: https://doi.org/10.1289/EHP7484

Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ (2009) Azole resistance in *Aspergillus fumigatus:* a side-effect of environmental fungicide use? Lancet Infect Dis 9: 789–795. doi: 10.1016/S1473-3099(09)70265-8

WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO

Chapter 1:

Literature Review: Yeast contaminated water and its emerging health concern

(This review paper has been accepted for publication)

Baker T, Albertyn J, Musoke J, Sebolai O, Pohl CH (2024) Yeast-contaminated water as a potential emerging health concern: a review. Water SA, 50(4), pp.404-410. doi: https://doi.org/10.17159/wsa/2024.v50.i4.4097

Abstract

Considering the emerging concern posed by invasive fungal infections, it is important to study the dissemination and proliferation of pathogenic fungal species in the environment. It is crucial to identify major vectors that aid in the spread or act as a hub for pathogenic yeasts in order to prevent infections in susceptible individuals, which mainly include individuals who are immunocompromised or immunosuppressed. *Candida*, *Cryptococcus* and *Rhodotorula* species are commonly found in a variety of water sources with which humans are in frequent contact through daily activities like bathing, washing clothes and cooking. The World Health Organisation has recently published a list of priority pathogens in which fungi are classified into critical, high and medium-priority groups. *Candida* species are found in all three levels and *Cryptococcus* species are in critical and medium groups. This further warrants the investigation into the possibility that infections may occur through contact with yeast-contaminated water. In addition, the close association between antifungal pollutants and yeast in water may induce antifungal resistance development, further complicating the effective treatment of these infections. Thus, it is important to investigate the presence and antifungal susceptibility of yeast found in water as well as to identify ways to monitor potential fungal outbreaks, including through wastewater surveillance. This review deals with the occurrence and infection risks posed by pathogenic yeasts in water as well as the possibility of these yeasts acquiring antifungal resistance due to the simultaneous presence of antifungal compounds from medical and agricultural runoff.

Keywords: Pathogenic yeast; Water quality; Antifungal resistance; Azoles; Wastewater surveillance

Introduction

Humans can have direct or indirect contact with water-borne fungi that may include pathogenic yeast species. These yeast species pose health risks to individuals who have weakened immune systems (Alcazar-Fuoli and Mellado, 2014; Bongomin et al., 2017; Parslow and Thornton, 2022; Pour et al., 2018) in the form of invasive fungal infections, which according to recent estimates, account for 3.8 million deaths annually (Denning, 2024). Considering the threat that opportunistic pathogenic yeasts hold, it is imperative to investigate how these organisms spread and proliferate in environments that humans are in frequent contact with. Potentially pathogenic yeast species have been identified in various water sources, including wastewater, drinking water and seawater (Ayanbimpe et al., 2012; Evans and Seviour, 2012; Pour et al., 2018; Pereira et al., 2009; Yamasato et al., 1974; Yang et al., 2011). Their presence in water may be due to the shedding of these organisms from the human microbiome or through the cleaning of household surfaces (Adams et al., 2013). Interestingly, potentially pathogenic yeast species belonging to genera such as *Candida, Rhodotorula* and *Cryptococcus* seem to be common in all the above-mentioned water sources as well as in the treated water from wastewater treatment plants (WWTPs) (Kacprzak et al., 2005; Monapathi et al., 2021a; Monapathi et al., 2021b; Pereira et al., 2009; Yamaguchi et al., 2007; Yang et al., 2013). This indicates the lack of effective removal of fungal species by current wastewater treatment methods.

Fungi are also overlooked in the regulation of water quality (Sonigo et al., 2011), further supporting the hypothesis that the vast network of connected water sources contributes to the dissemination of pathogenic yeasts. An additional concern is the development of antifungal resistance due to environmental pollution by antifungals. Antifungals are commonly found in water due to fungicide runoff from the agricultural industry (Dalhoff, 2017), personal care products (e.g., shampoos and topical ointments) being washed down the drain (Liu et al., 2016; Yao et al., 2016) and the excretion of antifungal medication by humans into wastewater systems (Kahle et al., 2008). Previous studies showed that WWTPs collect antibiotics, antibiotic-resistant bacteria, as well as antimicrobial resistance genes and that this close association of resistance components may lead to the development of antibiotic-resistant bacteria (Osińska et al., 2020; Turolla et al., 2018). It is theorised that the association between yeast and antifungals in the environment may also lead to the acquired resistance through constant exposure to low levels of antifungals (Fischer et al. 2022). Finding ways to monitor the presence of multidrug-resistant yeast species may help the health sector prepare for and treat fungal infections more effectively and efficiently.

This review aims to highlight the occurrence of potentially pathogenic yeasts in various water sources as well as the risk for the development of antifungal resistance within these habitats. We also advocate for increased surveillance of these emerging pathogens.

Fungal infection incidence and severity

Fungal infections, including yeast infections, affect more than a billion people and cause approximately 3.8 million deaths annually (Denning, 2024). The major driver in fungal infection incidence is the increase in cases of cancer, asthma, HIV/AIDS, chronic obstructive pulmonary disease and tuberculosis (Bongomin et al., 2017; Pour et al., 2018). Other causes of invasive fungal infections include the use of more aggressive therapeutic approaches (e.g., chemotherapy, immunotherapy, organ transplants) that lead to patients being in a prolonged state of immunosuppression (Alcazar-Fuoli and Mellado, 2014) and other underlying health conditions which increase the probability of opportunistic infection (Parslow and Thornton, 2022).

Invasive candidiasis (including candidemia) is a common fungal infection caused by yeast species from the *Candida* genus, with an estimated occurrence of 1 500 000 cases per year (Figure 1.1). Five *Candida* species, namely *Candida albicans, Candida glabrata, Pichia kudriavzevii* (anamorph = *Candida krusei*)*, Candida tropicalis* and *Candida parapsilosis,* cause more than 90% of these cases (Parslow and Thornton, 2022) with *C. albicans* being the predominant causative agent (Pfaller et al., 2007). Another *Candida* species which is also considered to be an emerging nosocomial pathogen with global occurrence, is *Candida auris* (Al-Rashdi et al., 2021; Du et al., 2020). Reports indicate that *C. auris* is associated with high mortality rates (30-60%), especially in immunocompromised individuals (CDC, 2019).

Even though literature frequently highlights the severity of candidiasis, these infections are still neglected by public health authorities (Parslow and Thornton, 2022). This has prompted the World Health Organisation (WHO) to publish the first Fungal Priority Pathogens list, which includes *Candida* spp. among the critical and high priority groups (WHO, 2022).

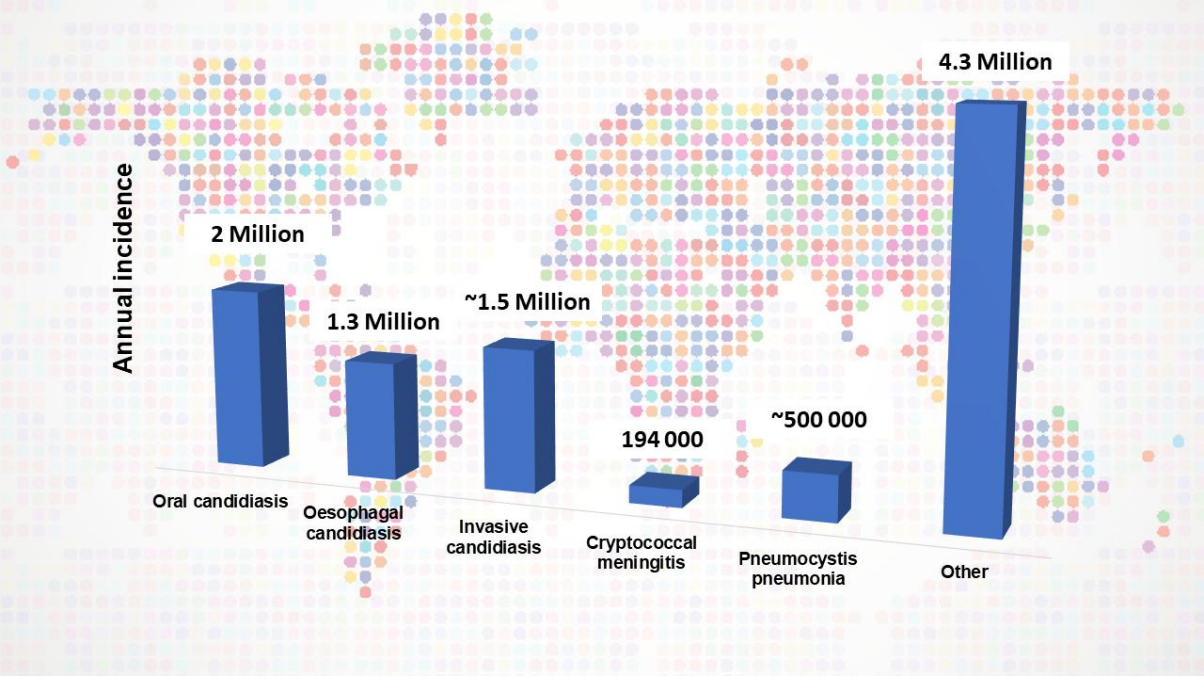


Figure 1.1. Annual incidence of fungal infections [adapted from Denning (2024)]

Other yeast genera containing species capable of causing invasive fungal infections include *Cryptococcus* and *Rhodotorula*, which are also frequently isolated from the aquatic environment. According to the National Institute of Communicable Diseases (NICD, 2022), *Cryptococcus* is the leading cause of meningitis as well as the second leading cause of death among adults living with HIV in low- and middle-income countries (Rajasingham et al., 2022). It is estimated that there are 194 000 annual cases of cryptococcal meningitis that result in 118 000 directly attributable cryptococcal-related deaths globally (Denning, 2024). *Cryptococcus neoformans* was also recently included in the critical priority group of the Fungal Priority Pathogens group by the WHO (WHO, 2022). *Rhodotorula spp.* are currently considered emerging pathogens, mainly infecting immune-compromised individuals (Gomez-Lopez et al., 2005; Jarros et al., 2020). Members of this genus show resistance against azoles and echinocandins, limiting available treatment options, and in China, this yeast is the main non-*Candida* causative agent for invasive fungal infections (Chen et al., 2021; Xiao et al., 2018). Infections by *Rhodotorula* can cause systemic, central nervous system, ocular and intraabdominal infections, with *Rhodotorula mucilaginosa* being the most common causative agent (Ferreira et al., 2022).

Risk of yeast exposure through water

Approximately 25% of people worldwide lack access to basic drinking water management services (UN Water, 2021). The countries with the lowest access are those in sub-Saharan Africa and Oceania.

Furthermore, 35% of the 2 billion people without access to basic sanitation live in sub-Saharan Africa (WHO and UNICEF, 2019), with 37% of households in South Africa still without access to water-borne sewage systems (StatsSA, 2017). These households still make use of non-sewage sanitation systems like pit, bucket and chemical toilets. Pit latrines are typically not isolated from the surrounding soil, thus water from these latrines can seep into groundwater. Heavy rainfall can also cause pit latrines to overflow, placing surrounding residents in danger of exposure to possible pathogens (Graham and Polizzotto 2013; Sengupta et al., 2018). This makes it important to assess the risk of contracting invasive fungal infections from water polluted by opportunistic or pathogenic yeasts.

Steffen and colleagues (2023) aimed to shed light on the risk of contracting invasive yeast infections by developing a Quantitative Microbial Risk Assessment framework to determine the potential risk of yeast infection from consuming water from high and low-polluted water samples from river water in Stellenbosch, South Africa. This framework considered variables such as the pathogenic potential of the species, based on clinical data considering the fraction of symptomatic infections, the infecting inoculum and the fraction of deaths from infection, as well as the estimated dose that will be received during both accidental and intentional ingestion of the water. Several assumptions were made during the establishment of the framework, including that people ingest yeast-containing water every day; that all strains of a species react similarly when ingested; that 37^{o}C mimics the stress a yeast will be exposed to in a host; that translocation from the intestines to the bloodstream happens post-ingestion and that the murine model mimics human host. This framework was applied to six clinically relevant yeast species (*C. albicans, C. tropicalis, C. glabrata, Candida lusitaniae*, *Meyerozyma guilliermondii* (anamorph = *Candida guilliermondii*) and *P. kudriavzevii*). In highly polluted water, *C. albicans* and *C. tropicalis* had the highest annual probability of causing infection via ingestion. *C. glabrata* had the highest risk assessment of strains detected in both highly polluted and less polluted samples. Although the risk of *C. neoformans* infection from water was not assessed and may indeed be low due to infection being primarily via inhalation, it is known that direct inoculation into tissues is possible (Christianson et al. 2003). In addition, *C. neoformans* can occasionally cause gastrointestinal infection (Maziarz and Perfect, 2026; Naranjo-Saltos et al., 2020). Thus, water contaminated with pathogenic yeast may pose a health risk to communities that come into contact with them, although for *C. neoformans* more research into the possible risk is needed.

How common are pathogenic yeasts in water?

Fungi have been identified in various water sources, including the marine environment, freshwater sources, hospital water and WWTPs (Arvanitidou et al., 1999; Medeiros et al., 2012; Yamasato et al., 1974; Yang et al., 2011). Although yeasts are generally present in low numbers in water, they can reach significant densities. Hagler and Ahearn (1987) reported average counts of up to 50 colony forming units (CFUs)/liter in seawater, 100 CFUs/liter in lakes and 500 CFU/liter in rivers, while urban estuaries have been reported to contain 2800 CFUs/liter. It is also known that yeasts can survive for prolonged periods of time in water (Starmer and Lachance, 2011). For example, *R. mucilaginosa* has a mean survival of over 600 days in river water (Peter and Peter, 1988).

Recent studies have expanded our knowledge regarding the occurrence of pathogenic yeasts in various water sources. Fourteen rivers and four lakes from the Doce River basin in Brazil were studied (Medeiros et al., 2012) and *Candida* spp. including the opportunistic pathogens *M. guilliermondii, C. parapsilosis* and *P. kudriavzevii* were found. Similar results were also found in surface water from the North-West province, South Africa (Monapathi et al., 2021a). Here *Candida* was also the dominant genus, with *C. tropicalis* the most abundant species in all the sampling sites in this study.

Pereira and co-workers (2009) investigated the presence of yeasts in three different drinking water sources (surface, spring and groundwater) in Portugal. Various *Candida* spp. were also identified in bottled mineral water dispensers and municipally supplied tap water in Brazil, with *C. parapsilosis* most frequently isolated, followed by *C. glabrata* and *C. albicans* (Yamaguchi et al., 2007). Similarly, a study done on 150 water samples, from Jos, Nigeria, identified *C. tropicalis* as the most prominent species in the water samples (Ayanbimpe et al., 2012). Investigation of potable water from hospitals and community taps in Thessaloniki, Greece (Arvanitidou et al., 1999), as well as biofilms in water distribution systems in Springfield, Missouri (Doggett 2000), identified species of *Candida* and *Rhodotorula*. In addition, Novak Babič and co-workers (2016) found that ground and tap water contain various opportunistic yeasts. including *R. mucilaginosa, C. parapsilosi*s, and *M. guilliermondii.*

Numerous studies point to sewage acting as an ideal medium to support the growth of various filamentous fungi as well as yeast (Cooke 1970; Cooke and Pipes 1969; Fakhru'l-Razi and Molla 2007; Kacprzak et al., 2005), where they may play a role in degradation of polysaccharides and production of secondary metabolites, that may influence the surrounding bacterial community. Importantly,

some of the yeasts in activated sludge are known pathogens, including *C. albicans* and *P. kudriavzevii* (Cooke 1970; Cooke and Pipes 1969; Kacprzak et al., 2005).

From all these studies it can be concluded that water frequently contains pathogenic yeast species, yet the inclusion of fungal regulation in water quality assessments is still sparse. For instance, the South African Water Quality Guidelines (Department of Water Affairs and Forestry, 1996) do not regulate the fungal load in treated water. Given that there is a risk of infection due to ingestion of water containing some of these yeasts, further research is needed regarding the need for regulation.

Antifungals present in water and associated resistance

Antifungals are frequently found in the environment due to use in pharmaceutical, veterinary and agricultural industries. Azoles are the most commonly used antifungals in the treatment of superficial and invasive mycoses (Vanreppelen et al., 2023), and they are used in agricultural fungicides (Hof 2001; Li et al., 2019; Toda et al., 2019). Antifungals are also used as food preservatives (Dalhoff 2017) and in personal care products (Liu et al., 2016; Yao et al., 2016). Thus, it is not surprising that they are pollutants in water sources such as freshwater, groundwater and WWTPs (Figure 1.2) (Assress et al., 2020; Chen et al., 2014; Huang et al., 2010; Huang et al., 2013; Lindberg et al., 2010; Liu et al., 2016; Monapathi et al., 2017; Monapathi et al., 2021b; Yao et al., 2016).

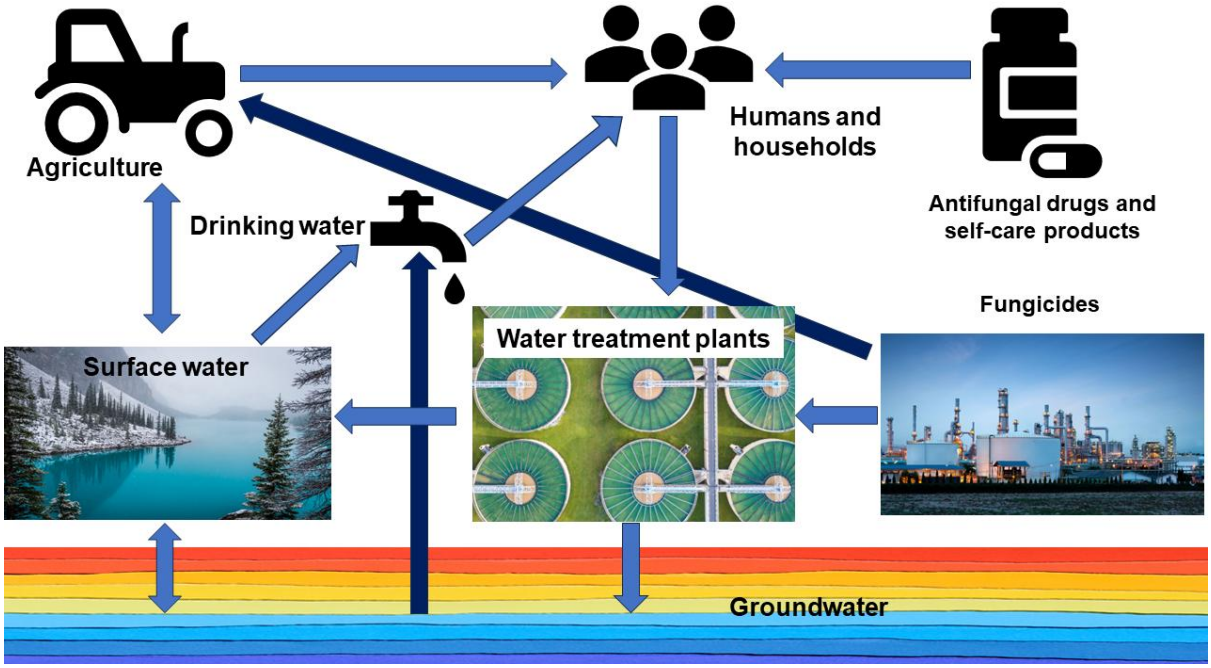


Figure 1.2. Route of transmission of antifungals from various sources to water. Antifungals enter water through waste discharge from industrial, hospital and domestic sources as well as from agricultural runoff. Humans have frequent contact with various of these antifungal polluted water sources through daily activities

Antifungals/fungicides identified in freshwater include fluconazole, econazole, miconazole, climbazole, flucytosine, ketoconazole, propiconazole, tebuconazole and carbendazim (Chen et al., 2014; Huang et al., 2013; Monapathi et al., 2021a; Monapathi et al., 2021b). Monapathi et al. (2021a) found a positive correlation between fluconazole concentration and the presence of *C. tropicalis*. To further support the idea of antifungal resistance originating in aquatic environments, Monapathi and co-workers (2017) tested antifungal resistance of previously isolated pathogenic yeasts sampled from the same freshwater sources in the Northwest Province of South Africa and found all the isolates to be resistant to miconazole and flucytosine. In addition, 88.5% of isolates were resistant to fluconazole and econazole, 62.8% were resistant to miconazole, and 64.1% were resistant to ketoconazole.

Similar to fresh water, antifungals including clotrimazole, econazole, fluconazole, itraconazole, ketoconazole, posaconazole and miconazole are repeatedly found in WWTPs (Assress et al., 2019; Assress et al., 2020; Huang et al., 2012; Kahle et al., 2008; Peng et al., 2012), with fluconazole being the most abundant azole in wastewater influent and effluent (Assress et al., 2019; Assress et al., 2020). WWTPs can function as important hubs that aid in the dissemination of bacterial resistance genes into environmental waters. This is frequently seen in the identification of antibiotic resistant bacteria in treated and untreated wastewater as well as surrounding waters (Alexander et al., 2020; Kotlarska et

al., 2015; Osińska et al., 2020). Many studies concluded that WWTPs have become a collection area for antibiotics, antibiotic-resistant bacteria as well as resistant genes and that treatment plants contribute to the development of antimicrobial resistance in bacteria (Birošová et al., 2014; Naquin et al., 2015; Osińska et al., 2020; Turolla et al., 2018). However, few reports are available on how the presence of antifungals in WWTP can contribute to the development of antifungal resistance, although the occurrence of both fungi and antifungal drugs may result in conditions which are favourable for development of acquired resistance in fungi (Fischer et al., 2022). Using the risk quotient method, Assress et al. (2020) determined that the risk for selection of resistance to the azoles by fungi was moderate to high for fluconazole and itraconazole in the studied South African WWTPs.

Wastewater surveillance and its application

Wastewater surveillance, or wastewater-based epidemiology (WBE), is the monitoring of wastewater for agents of interest, such as viruses, that could indicate a possible disease outbreak and overall health of a community. It is well documented that monitoring wastewater can help to determine the potential disease burden of a community (Brumfield et al., 2022; McCall et al., 2020) since pathogens are frequently shed by humans during active infection (Feachem et al., 1983; Sinclair et al., 2008). In addition, WBE can also point to possible future outbreaks, aiding healthcare professionals to prepare accordingly (McCall et al., 2020). Clinical data on disease incidence only cover a select proportion of the population who are admitted to or voluntarily go to the hospital, while WBE assesses the complete community of an area, including individuals who prefer home care or who cannot afford to go to hospitals. Thus, it is more accurate and less biased as an indicator on the overall health of a community. Figure 1.3 depicts the basic workflow of wastewater surveillance.

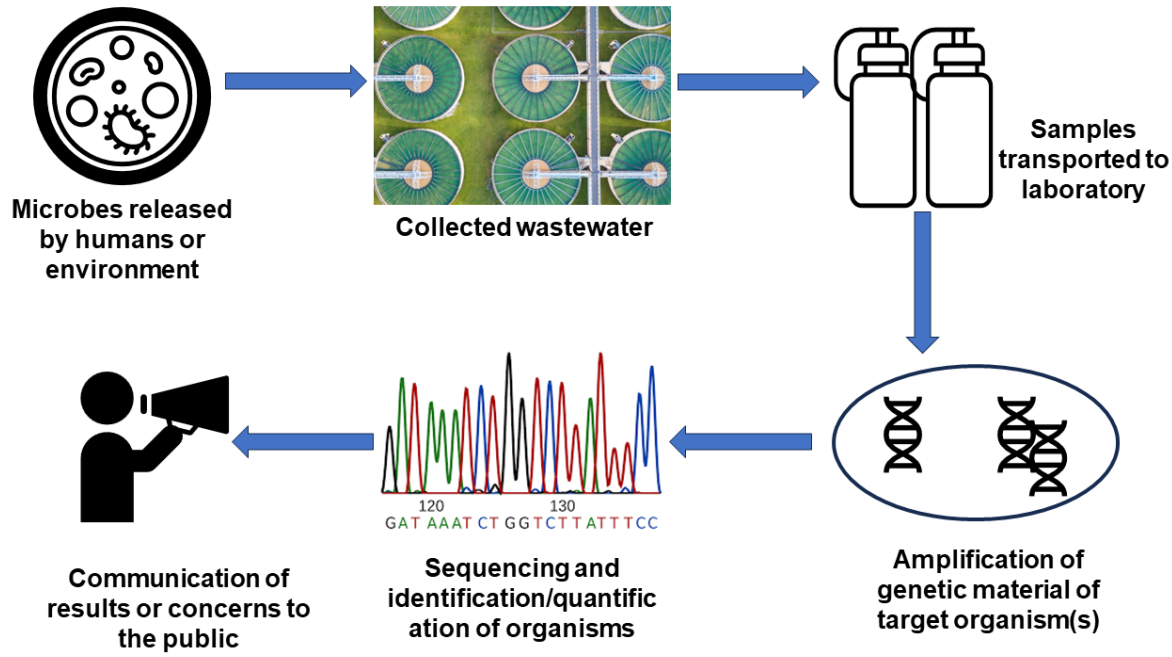


Figure 1.3. Basic workflow of wastewater surveillance. Wastewater is transported to a central location for treatment, samples are collected at these locations or any other sewage access point according to a method the researchers deem most fit. Collection is followed by processing and the extraction of genetic material, either RNA or DNA, from the samples in a laboratory. After extraction, biomarkers in genetic material are amplified and identified using reverse transcription–polymerase chain reaction and next-generation genomic sequencing to identify pathogens or other target microorganisms. Data obtained are then communicated to the public.

Previously wastewater surveillance has been used to monitor poliomyelitis outbreaks in Charlestown, North Carolina (Paul et al., 1939) and in recent times this method has been used to detect vaccine-derived poliovirus in the sewage of north and east London as well as detection of wild poliovirus type 1 in Israel, before the virus caused any cases of paralysis (Anis et al., 2013; Wise 2022). Another recent application of WBE is in the monitoring of SARS-CoV-2 viral loads to predict COVID-19 outbreaks in specified areas (Brumfield et al., 2022; Hart and Halden 2020; Peccia et al., 2020; Wu et al., 2021). WBE has also detected various bacteria, including human pathogens and detection of coliforms present in WWTPs are essential for the evaluation of the efficiency of treatment procedures.

Although WBE seems to be ideal for use as early warning system and to provide reliable and rapid information regarding the health of a community, there are limitations to this method. These include cost and logistics, complexity of wastewater matrix (including the presence of PCR inhibitors) (Walden et al., 2017) and dilution and stability of biomarkers (Mao et al., 2020). Applying this system to the identification of pathogenic yeast may also present unique challenges. One of these challenges are

that fungal species can only be identified if the genome database used contains the specific sequence (Ryberg and Nilsson 2018). Since fungal genome databases still have limited reference sequences, this can lead to the false identification of yeast. Despite these challenges, recent attempts at targeted WBE for the important emerging pathogen, *C. auris* have shown promise (Babler et al., 2023; Barber et al., 2023; Rossi et al., 2023; Zulli et al., 2024), laying the foundations for future application of this system for other pathogenic yeasts.

Conclusions

Pathogenic yeasts are commonly found in various water sources with which humans and animals are in frequent contact. Considering that yeasts are cultured from the effluent of WWTPs, the lack of regulations regarding the presence of fungi in treated wastewater and the insufficiency of wastewater treatment processes is evident. In addition, the presence of pathogenic yeast strains in drinking water does pose a risk of infection as shown for C. albicans and C. kudriavzevii. These yeasts may be disseminated via water transport systems, thus allowing them to reach a larger proportion of the population with both healthy and susceptible individuals.

The presence of yeast in water along with the pollution of water sources with antifungals originating from the pharmaceutical and cosmetic industries and agricultural runoff are cause for concern due to the possibility of antifungal resistance development. Taking this into consideration, monitoring possible multidrug-resistant yeast in the environment can prove useful in preparing the healthcare sector for the potential occurrence of fungal infections with otherwise unexpected antifungal resistance profiles.

Wastewater surveillance has proven useful in the past in the monitoring polio outbreaks as well as in the detection of possible COVID-19 hotspots so if this tool could be applied to the monitoring of pathogenic yeast, it could be valuable. The application of this tool for this use seems promising and should be further investigated to confirm its efficiency and accuracy.

References

Adams RI, Miletto M, Taylor JW, Bruns TD (2013) The diversity and distribution of fungi on residential surfaces. PLOS ONE **8:** e78866. doi: https://doi.org/10.1371/journal.pone.0078866

Alcazar-fuoli L, Mellado E (2014) Current status of antifungal resistance and its impact on clinical practice. Br J Haematol 166: 471-484. doi: https://doi.org/10.1111/bjh.12896

Alexander J, Hembach N, Schwartz T (2020) Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci Rep 10**:** 8952. doi: https://doi.org/10.1038/s41598-020-65635-4

Al-rashdi A, Al-maani A, Al-wahaibi A, Alqayoudhi A, Al-jardani A, Al-abri S (2021) Characteristics, risk factors, and survival analysis of *Candida auris* cases: Results of one-year national surveillance data from Oman. J Fungi 7**:** 31. doi: https://doi.org/10.3390/jof7010031

Ayanbimpe GM, Abbah VE, Ior CA (2012) Yeasts and yeast-like fungal contaminants of water used for domestic purposes in Jos, Nigeria. Microbiol Res 3**:** e24. doi: https://doi.org/10.4081/mr.2012.e24

Anis E, Kopel E, Singer SR, Kaliner E, MoermaN L, Moran-gilad J, Sofer D, Manor Y, Shulman LM, Medelson E, Gdalevich M, Lev B, Gamzu R, Grotto I (2013) Insidious reintroduction of wild poliovirus into Israel. Eurosurveillance 18: 20586. doi: https://doi.org/10.2807/1560-7917.ES2013.18.38.20586

Arvanitidou M, Kanellou K, Constantinides TC, Katsouyannopoulos V (1999) The occurrence of fungi in hospital and community potable waters. Lett Appl Microbiol 29**:** 81–84. doi: https://doi.org/10.1046/j.1365-2672.1999.00583.x

Assress HA, Nyoni H, Mamba BB, Msagati TAM (2020) Occurrence and risk assessment of azole antifungal drugs in water and wastewater. Ecotoxicol Environ Saf 187: 109868. doi: https://doi.org/10.1016/j.ecoenv.2019.109868

Assress HA, Selvarajan R, Nyoni H, Ntushelo K, Mamba BB, Msagati TAM (2019) Diversity, co-occurrence and implications of fungal communities in wastewater treatment plants. Sci Rep 9: 14056. doi: https://doi.org/10.1038/s41598-019-50624-z

Babler, K., Sharkey, M., Arenas, S., Amirali, A., Beaver, C and co-workers (2023) Detection of the clinically persistent, pathogenic yeast spp. *Candida auris* from hospital and municipal wastewater in Miami-Dade County, Florida. Sci Total Environ 898: 165459. doi: 10.1016/j.scitotenv.2023.165459

Barber C, Crank K, Papp K, Innes GK, Schmitz BW, Chavez J, Rossi A, Gerrity D (2023) Community-scale wastewater surveillance of *Candida auris* during an ongoing outbreak in southern Nevada. Environ Sci Technol 57: 1755-1763. doi: https://doi.org/10.1021/acs.est.2c07763

Birošová L, Mackuľak T, Bodík I, Ryba J, Škubák J, Grabic R (2014) Pilot study of seasonal occurrence and distribution of antibiotics and drug resistant bacteria in wastewater treatment plants in Slovakia. Sci Total Environ 490**:** 440-444. doi: https://doi.org/10.1016/j.scitotenv.2014.05.030

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases – estimate precision. J Fungi 3: 57. doi: https://doi.org/10.3390/jof3040057

Brumfield KD, Leddy M, Usmani M, Cotruvo JA, Tien CT, Dorsey S, Graubics K, Fanelli B, Zhou I, Registe N, Dadlani M (2022) Microbiome analysis for wastewater surveillance during COVID-19. Mbio 13: e00591-22. doi: https://doi.org/10.1128/mbio.00591-22

CDC (2019) About *Candida auris*. Retrieved 31 March 2023 from https://www.cdc.gov/fungal/candida-auris/candida-auris-qanda.html

Chen SCA, Perfect J, Colombo AL, Cornely OA, Groll AH, Seidel D, Albus K, de Almeida J, Garcia-Effron G, Gilroy N and co-workers (2021) Global guideline for the diagnosis and management of rare yeast infections: an initiative of the ECMM in cooperation with ISHAM and ASM. Lancet Infect Dis 21: e375–386. doi: https://doi.org/10.1016/S1473-3099(21)00203-6

Chen ZF, Ying GG, Liu YS, Zhang QQ, Zhao JL, Liu SS, Chen J, Peng FJ, Lai HJ, Pan CG (2014) Triclosan as a surrogate for household biocides: an investigation into biocides in aquatic environments of a highly urbanized region. Water Res 58: 269e279. doi: https://doi.org/10.1016/j.watres.2014.03.072

Christianson JC, Engber W, Andes D (2003) Primary cutaneous cryptococcosis in immunocompetent and immunocompromised hosts. Med Mycol 41: 177-188. doi: 10.1080/1369378031000137224

Cooke WB (1970) Fungi associated with the activated-sludge process of sewage treatment at the Lebanon, Ohio, sewage treatment plant. Ohio J Sci 70: 129–146.

Cooke WB, Pipes WO (1969) The occurrence of fungi in activated sludge. Mycopathologia 40: 249–270. doi: https://doi.org/10.1007/BF02051779

Dalhoff A (2017) For debate: May the use of the polyene macrolide natamycin as a food additive foster emergence of polyene-resistance in *Candida* species? Clin Microbiol 6: 4. doi: https://doi.org/10.4172/2327-5073.1000287

Denning DW (2024) Global incidence and mortality of severe fungal disease. Lancet Infect Dis 24: E428-E438. doi: 10.1016/S1473-3099(23)00692-8

Department of water affairs and forestry (1996) South African Water Quality Guidelines Volume 1: Domestic Water Use (2nd ed)

Doggett MS (2000) Characterization of fungal biofilms within a municipal water distribution system. Appl Environ Microbiol 66: 1249-1251. doi: https://doi.org/10.1128/aem.66.3.1249-1251.2000

Du H, Bing J, Hu T, Ennis CL, Nobile CJ, Huang G (2020) *Candida auris*: Epidemiology, biology, antifungal resistance, and virulence. PLoS Path 16: e1008921. doi: https://doi.org/10.1371/ journal.ppat.1008921

Evans TN, Seviour RJ (2012) Estimating biodiversity of fungi in activated sludge communities using culture-independent methods. Microb Ecol 63: 773-786. doi: https://doi.org/10.1007/s00248-011-9984-7

Ferreira AI, Cruz H, Ranchor R, Silva BS, Serôdio J, Lopes V, Ramos MH (2022) Pleural empyema due to *Rhodotorula mucilaginosa*: A rare yet severe complication of a previously undiagnosed cancer patient. IDCases 28: e01469. doi: https://doi.org/10.1016/j.idcr.2022.e01469

Fakhru'L-Razi A, Molla AH (2007) Enhancement of bioseparation and dewaterability of domestic wastewater sludge by fungal treated dewatered sludge. J Haz Mat 147: 350–356. doi: https://doi.org/10.1016/j.jhazmat.2007.01.060

Feachem RG, Bradley DJ, Garelick H, Mara DD (1983) *Sanitation and disease: health aspects of excreta and wastewater management.* John Wiley and Sons, New York. 9 pp.

FIscher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, Bromley M, Brüggemann R, Garber G, Cornely OA, Gurr SJ, Harrison TS, Kuijper E, Rhodes J, Sheppard DC, Warris A, White PL, XU J, Zwaan B, Verweij PE (2022) Tackling the emerging threat of antifungal resistance to human health. Nat Rev Microbiol 20: 557-571. doi: 10.1038/s41579-022-00720-1

Gomez-Lopes A, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M (2005) Susceptibility profile of 29 clinical isolates of *Rhodotorula* spp. and literature review. J Antimicrob Chemother 55: 312-318. doi: https://doi.org/10.1093/jac/dki020

Graham JP, Polizzotto ML (2013) Pit latrines and their impacts on groundwater quality: a systematic review. Environ Health Perspect 121: 521-530. doi: http://dx.doi.org/10.1289/ehp.1206028

Hagler AN, Ahearn DG (1987) Ecology of aquatic yeasts. In: Rose AH and Harrison JS (eds.) The Yeasts Volume 1 (2nd ed) Academic Press., Cambridge, Massachusetts, USA.

Hart OE, Halden RU (2020) Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy, opportunities and challenges. Sci Total Environ 730: 138875. doi: https://doi.org/10.1016/j.scitotenv.2020.138875

Hof H (2001) Critical annotations to the use of azole antifungals for plant protection. Antimicrob Agents Chemother 45: 2987-2990. doi: https://doi.org/10.1128/AAC.45.11.2987-2990.2001

Huang Q, Wang Z, Wang C, Peng X (2013) Chiral profiling of azole antifungals in municipal wastewater and recipient rivers of the Pearl River Delta, China. Environ Sci Poll Res 20: 8890-8899. doi: https://doi.org/10.1007/s11356-013-1862-z

Huang Q, Yu Y, Tang C, Peng X (2010) Determination of commonly used azole antifungals in various waters and sewage sludge using ultra-high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1217: 3481-3488. doi: https://doi.org/10.1016/j.chroma.2010.03.022

Huang Q, Zhang K, Wang Z, Wang C, Peng X (2012) Enantiomeric determination of azole antifungals in wastewater and sludge by liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 403: 1751–1760. doi: https://doi.org/10.1007/s00216-012-5976-9

Jarros IC, Veiga FF, Corrêa JL, Barros ILE, Gadelha MC, VoidaleskI MF, Pieralisi N, Pedroso RB, Vicente VA, Negri M, Svidzinski TIE (2020) Microbiological and virulence aspects of *Rhodotorula mucilaginosa*. EXCLI J 19: 687-704. doi: http://dx.doi.org/10.17179/excli2019-1672

Kacprzak M, Neczaj E, Okoniewska E (2005) The comparative mycological analysis of wastewater and sewage sludges from selected wastewater treatment plants. Desalination 185: 363–370. doi: https://doi.org/10.1016/j.desal.2005.03.085

Kahle M, Buerge IJ, Hauser A, Muller MD, Poiger T (2008) Azole fungicides: occurrence and fate in wastewater and surface waters. Environ Sci Technol 42: 7193-7200. doi: https://doi.org/10.1021/es8009309

Kotlarska E, Łuczkiewicz A, Pisowacka M, Burzyński A (2015) Antibiotic resistance and prevalence of class 1 and 2 integrons in *Escherichia coli* isolated from two wastewater treatment plants, and their receiving waters (Gulf of Gdansk, Baltic Sea, Poland). Environ Sci Pollut Res 22: 2018-2030. doi: https://doi.org/10.1007/s11356-014-3474-7

Li S, Wu Q, Sun Q, Coffin S, Gui W, Zhu G (2019) Parental exposure to tebuconazole causes thyroid endocrine disruption in zebrafish and developmental toxicity in offspring. Aquat Toxicol 211: 116-123. doi: https://doi.org/10.1016/j.aquatox.2019.04.002

Lindberg RH, FIck J, Tysklind M (2010) Screening of antimycotics in Swedish sewage treatment plants – Waters and sludge. Water Res 44**:** 649-657. doi: https://doi.org/10.1016/j.watres.2009.10.034

Liu J, Lu G, Yang H, Yan Z, Wang Y, Wang P (2016) Bioconcentration and metabolism of ketoconazole and effects on multi-biomarkers in crucian carp (*Carassius auratus*). Chemosphere 150: 145-151. doi: https://doi.org/10.1016/j.chemosphere.2016.02.033

MaziARZ EK, Perfect JR (2026) Cryptococcosis. Infect Dis Clin North Am 30: 179-206. doi: 10.1016/j.idc.2015.10.006

Mao K, Zhang K, Du W, Ali W, Feng X, Zhang H (2020) The potential of wastewater-based epidemiology as surveillance and early warning of infectious disease outbreaks. Curr Opin Environ Sci Health 17: 1-7. doi: https://doi.org/10.1016/j.coesh.2020.04.006

Mccall C, Wu H, Miyani B, Xagoraraki I (2020) Identification of multiple potential viral diseases in a large urban center using wastewater surveillance. Water Res 184: 116160. doi: https://doi.org/10.1016/j.watres.2020.116160

Medeiros AO, Missagia BS, Brandão LR, Callisto M, Barbosa FAR, Rosa CA (2012) Water quality and diversity of yeast from tropical lakes and rivers from the Rio Doce basin in Southeastern Brazil. Braz J Microbiol 43**:** 1582-1594. doi: https://doi.org/10.1590/S1517-83822012000400043

Monapathi ME, Bezuidenhout CC, Rhode OHJ (2017) Water quality and antifungal susceptibility of opportunistic yeast pathogens from rivers. Water Sci Technol 75: 1319–1331. https://doi.org/10.2166/wst.2016.580

Monapathi ME, Horn S, Vogt T, Van Wyk D, Mienie C, Ezeokoli OT, Coertze R, Rhode O, Bezuidenhout CC (2021a). Antifungal agents, yeast abundance and diversity in surface water: Potential risks to water users. Chemosphere 274: 129718. doi: https://doi.org/10.1016/j.chemosphere.2021.129718

Monapathi ME, Oguegbulu JC, Adogo L, Klink M, Okoli B, Mtunzi F, Modise JS (2021b) Pharmaceutical pollution: Azole antifungal drugs and resistance of opportunistic pathogenic yeasts in wastewater and environmental water. Appl Environ Soil Sci 2021: 9985398. doi: https://doi.org/10.1155/2021/9985398

Naquin A, Shrestha A, Sherpa M, Nathaniel R, Boopathy R (2015) Presence of antibiotic resistance genes in a sewage treatment plant in Thibodaux, Louisiana, USA. Bioresour Technol 188: 79–83. doi: http://dx.doi.org/10.1016/j.biortech.2015.01.052

Naranjo-Saltos f, Hallo A, Hallo c, Mayancela A, Rojas A (2020) Gastrointestinal cryptococcosis associated with intestinal lymphangiectasia. Case Rep Med 2020: 787054

NICD (2022) HIV-associated cryptococcal meningitis: study finds reduced mortality in patients treated with flucytosine-based treatment. Retrieved 19 January 2023 from https://www.nicd.ac.za/hiv-associated-cryptococcal-meningitis-study-finds-reduced-mortality-in-patients-treated-with-flucytosine-based treatment.

Novak Babič MN, Zalar P, Zenko B, Džeroski S, Gunde-Cimerman N (2016) Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances. Fungal Ecol 20: 30-39. doi: http://dx.doi.org/10.1016/j.funeco.2015.10.001

Osińska A, Korzeniewska E, Harnisz M, Felis E, Bajkacz S, Jachimowicz P, Niestępski S, Konopka I. (2020) Small-scale wastewater treatment plants as a source of the dissemination of antibiotic resistance genes in the aquatic environment. J Haz Mat 381: 121221. doi: https://doi.org/10.1016/j.jhazmat.2019.121221

Parslow BY, Thornton CR (2022) Continuing shifts in epidemiology and antifungal susceptibility highlight the need for improved disease management of invasive candidiasis. Microorganisms 10: 1208. doi: https://doi.org/10.3390/microorganisms10061208

Paul JR, Trask JD, Culotta CS (1939) Poliomyelitic virus in sewage. Science 90: 258-259.

Peccia J, Zulli A, Brackney DE, Grubaugh ND, Kaplan eh, Casanovas-Massana A, Ko AI, Malik AA, Wang D, Wang M and co-author (2020) Measurement of SARS-CoV-2 RNA in wastewater tracks community infection dynamics. Nature Biotechnol 38: 1164–1167. doi: https://doi.org/10.1038/s41587-020-0684-z

Peng X, Huang Q, Zhang K, Yu Y, Wang Z, Wang C (2012) Distribution, behavior and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci Total Environ 426: 311-7. doi: https://doi.org/10.1016/j.scitotenv.2012.03.067

Pereira VJ, Basílio MC, Fernandes D, Domingues M, Paiva JM, Benoliel MJ, Crespo MT, San Romão MV (2009) Occurrence of filamentous fungi and yeasts in three different drinking water sources. Water Res 43: 3813–3819. doi: https://doi.org/10.1016/j.watres.2009.05.024

Peter M, Peter Z (1988) Experimentelle studien bezueglich der persistenz einiger fakulativ pathogener pilze in flusswasser. Zentralblatt für Mikrobiologie 143: 523–528.

Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20: 133-163. doi: https://doi.org/10.1128/CMR.00029-06

Pour AH, Salari S, Almani PGN (2018) Oropharyngeal candidiasis in HIV/AIDS patients and non-HIV subjects in the Southeast of Iran. Curr Med Mycol 4: 1-6. doi: https://doi.org/10.18502/cmm.4.4.379

Rajasingham R, Govender NP, Jordan A, Loyse A, Shroufl A, Denning DW, Meya DB, Chiller TM, Boulware DR (2022) The global burden of HIV-associated cryptococcal infection in adults in 2020: a modelling analysis. Lancet Infect Dis 22: 1748-1755. https:/doi.org/10.1016/S1473-3099(22)00499-6

Rossi A, Chavez J, Iverson T, Hergert J, Oakeson K, Lacross N, Njoku C, Gorzalski A, Gerrity D (2023) *Candida auris* discovery through community wastewater surveillance during healthcare outbreak, Nevada, USA, 2022. Emerg Infect Dis 29: 422-425. doi: https://doi.org/10.3201/eid2902.221523

Ryberg M, Nilsson RH (2018) New light on names and naming of dark taxa. MycoKeys 23: 31-39. doi: https://doi.org/10.3897/mycokeys.30.24376

Sengupta S, Verma R, Kazmi S (2018) Bottom to the fore: State of sanitation in sub-Saharan Africa, Centre for Science and Environment.

Sinclair RG, Choi CY, Riley MR, Gerba CP (2008) Pathogen surveillance through monitoring of sewer systems. Adv Appl Microbiol 65: 249-69. doi: https://doi.org/10.1016/S0065-2164(08)00609-6

Sonigo P, de Toni PSA, Reilly K (2011) A review of fungi in drinking water and the implications for human health, DEFRA.

Starmer WT, Lachance MA (2011). Yeast ecology. In: *The yeasts, volume 1* (5th ed) Kurtzman CP, Fell JW, Boekhout T (eds) Elsevier, London, United Kingdom.

Steffen HC, Smith K, van Deventer C, WeiskergeR C, Bosch C, Brandão J, Wolfaardt G, Botha A (2023) Health risk posed by direct ingestion of yeasts from polluted river water. Water Res 231: 119599. doi: https://doi.org/10.1016/j.watres.2023.119599

STATSSA (2017) General Household Survey 2017. URL: https://www.statssa.gov.za/publications/P0318/P03182017.pdf (Accessed 23 March 2023)

Toda M, Beer KD, Kuivila KM, Chiller TM, Jackson BR (2019) Trends in agricultural triazole fungicide use in the nited States, 1992–2016 and possible implications for antifungal-resistant fungi in human disease. Environ Health Perspect. 129: 055001. doi: https://doi.org/10.1289/EHP7484

Turolla A, Cattaneo M, Marazzi F, Mezzanotte V, Antonelli M (2018) Antibiotic resistant bacteria in urban sewage: Role of full-scale wastewater treatment plants on environmental spreading. Chomosphere 191: 761-769. doi: https://doi.org/10.1016/j.chemosphere.2017.10.099

UN WATER (2021) Summary Progress Update 2021 – SDG 6 – Water and Sanitation for All. Version: July 2021. Geneva, Switzerland. URL: https://www.unwater.org/publications/summary-progress-update-2021-sdg-6-water-and-sanitation-all (Accessed 16 October 2023)

Vanreppelen G, Wuyts J, van DIjck P, Vandecruys P (2023) Sources of antifungal drugs. J Fungi 9: 171. doi: https://doi.org/10.3390/jof9020171

Walden C, Carbonero F, Zhang W (2017) Assessing impacts of DNA extraction methods on next generation sequencing of water and wastewater samples. J Microbiol Methods 141: 10-16. doi: http://dx.doi.org/10.1016/j.mimet.2017.07.007

WHO (2022) WHO fungal priority pathogens list to guide research, development and public health action. World Health Organization, Geneva, Switzerland.

WHO and UNICEF (2019) Progress on household drinking water, sanitation and hygiene 2000-2017: special focus on inequalities. Geneva: World Health Organization. URL: https://apps.who.int/iris/handle/10665/329370. (Accessed 23 October 2023)

Wise J (2022) Poliovirus is detected in sewage from north and east London. BMJ 2022: 377. doi: https://doi.org/10.1136/bmj.o1546

Wu F, Xiao A, Zhang J (2021) Wastewater surveillance of SARS-CoV-2 across 40 U.S. states from February to June 2020. Water Res 202: 117400. doi: https://doi.org/10.1016/j.watres.2021.117400

XIao M, Chen SCA, Kong F, Fan X, Cheng JW, Hou X, Zhou ML, Wang H, Xu YC (2018) Five-year China Hospital Invasive Fungal Surveillance Net (CHIF-NET) study of invasive fungal infections caused by noncandidal yeasts: species distribution and azole susceptibility. Infect Drug Resistn 11: 1659–67. doi: https://doi.org/10.2147/IDR.S173805

Yamaguchi MU, Rampazzo RCP, Yamada-Ogatta SF, Nakamura CV, Ueda-Nakamura T, Filho BPD (2007) Yeasts and filamentous fungi in bottled mineral water and tap water from municipal supplies. Braz Arch Biol Technol 50: 1-9. doi: https://doi.org/10.1590/S1516-89132007000100001

Yamasato K, Goto S, Ohwada K, Okuno D, Araki H, Iizuka H (1974) Yeasts from the Pacific Ocean. J Gen Appl Microbiol 20: 289-307. doi: https://doi.org/10.2323/jgam.20.289

Yang Q, Angly FE, Wang Z, Zhang H (2011) Wastewater treatment systems harbour specific and diverse yeast communities. Biochem Eng J 58-59: 168-176. doi: https://doi.org/10.1016/j.bej.2011.09.012

Yang Q, Zhang H, Li X, Wang Z, Xu Y, Ren S, Chen X, Xu Y, Hao H, Wang H (2013) Extracellular enzyme production and phylogenetic distribution of yeasts in wastewater treatment systems. Bioresour Technol 129: 264-273. doi: https://doi.org/10.1016/j.biortech.2012.11.101

Yao J, Zhao JL, Liu YS, Yang YY, Liu WR , Ying GG (2016) Simultaneous determination of 24 personal care products in fish muscle and liver tissues using QuEChERS extraction coupled with ultra pressure liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometer analyses. Anal Bioanal Chem 408: 8177–8193.

Zulli A, Chan EMG, Shelden B, Duong D, Xu X-RS, White BJ, Wolfe MK, Boehm AB (2024) Prospective study of *Candida auris* nucleic acids in wastewater solids in 190 wastewater treatment plants in the United States suggests widespread occurrence. Mbio 15: e0090824. doi: 10.1128/mbio.00908-24

Additional literature

The following literature was removed from the published version of the literature review.

Introduction

Wastewater surveillance is an early warning system for potential disease outbreaks in a catchment area. That entails monitoring wastewater for specific agents of interest, such as viral DNA, viral particles or bacteria (Janahi et al., 2020) to determine the disease burden of an area (Brumfield et al., 2022; McCall et al., 2020). In the past, this system has been used to monitor polio outbreaks (Paul et al., 1939) and more recently, to determine the prevalence of recent COVID-19 infections within a community (Hart and Halden 2020). Wastewater surveillance has proven to be an effective and efficient tool for the investigation of public health, yet few to no attempts have been made to apply this system to fungal infections. The potential application of this system to determine fungal infection burden seems promising but significant work still needs to be done to validate this tool for the specific application.

Fungal infection severity

During the recent COVID-19 pandemic, the incidences of fungal infections increased (Moser et al., 2021). Numerous patients, positive for infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), were diagnosed with fungal co-infections such as aspergillosis, mucormycosis and candidiasis (Hoenigl et al., 2022; Chen et al., 2020; Song et al., 2020). This occurrence is not only due to COVID-19 affecting the immune and metabolic response of humans but also due to the treatment associated with COVID-19 infections such as the use of corticosteroid therapy and mechanical ventilation (Ezeokoli et al., 2021; Hoenigl et al., 2022, Chen et al., 2020).

Moser and colleagues (2021) performed experimental work which aimed to investigate the immune phenotype and cytokine release pattern of patients admitted with COVID-19, as well as *ex vivo* experiments which revealed impaired immune response and increased susceptibility to *C. albicans* infections. Among patients with COVID-19-associated candidiasis, *C. albicans* was the most common, but infections by *C. glabrata* (Arastehfar et al., 2020; Chen et al., 2020; Seagle et al., 2022) and the multidrug-resistant *C. auris* (Arastehfar et al., 2020) have also been reported. This occurrence further

highlights the need for surveillance systems that aid in our knowledge of what fungal pathogens are circulating within a community.

Occurrence of yeast in wastewater

Activated sludge is involved in the biological treatment of wastewater. It is composed of a mixed community of microorganisms that aid in the degradation of various organic pollutants/matter as well as other toxic substances that may be present in sewage (More et al., 2010; Amann et al., 1998; Cooke and Pipes 1969). The role of fungi in activated sludge is yet to be elucidated, but it is proposed that they play a minor role since fungi are less abundant in activated sludge, compared to bacteria (Evans and Seviour 2012).

Numerous studies point to sewage acting as an ideal medium to support the growth of various filamentous fungi as well as yeast and yeast-like fungi (Fakhru'l-Razi and Molla 2007; Kacprzak et al., 2005; Cooke 1970; Cooke and Pipes 1969). Cooke and Pipes (1969) suggested that fungi and yeast in activated sludge function to degrade a variety of complex and simple polysaccharides and produce secondary metabolites like organic acid and antibiotics that may influence the surrounding bacterial community. Importantly, some of the yeasts in activated sludge are known pathogens, including species of *Trichosporon*, *Candida,* (including *C. albicans*)*, P. krudriavzevii*, *Rhodotorula, Torulopsis* and *Cryptococcus* (Kacprzak et al., 2005; Cooke 1970; Cooke and Pipes 1969).

More recent studies indicate that the major factors determining the fungal community present in activated sludge are the type of wastewater being treated as well as the treatment process used (Evans and Seviour 2012; Yang et al., 2011). In these studies, yeast from the genera *Candida, Pichia, Rhodotorula, Trichosporon* and unidentified ascomycetes were identified from different wastewater treatment systems (biopharmaceutical, paper and municipal). Studies by Yang and colleagues (2013) were done to derive the possible function of yeasts in wastewater treatment systems as well as the possible production of industrially important enzymes. They identified both ascomycetous yeast genera (*Torulaspora, Candida, Pichia, Dipodascus, Yarrowia, Sympodiomyces,* and *Geotrichum*) and basidiomycetous yeast genera (*Rhodotorula, Cryptococcus, Trichosporon, Pseudozyma*, and *Filobasidium*) in activated sludge. Some of these isolates had lipase, protease, lignin peroxidase and manganese-dependent peroxidase activity, indicating their possible application in other industry sectors.

From the literature, we can see that wastewater contains a variety of pathogenic and non-pathogenic yeast species that could either be shed from infected humans or from the surrounding environment.

It is also evident that current wastewater treatment procedures are not effective in the removal of fungi from waste influent.

Antifungals present in water and associated resistance

Antifungal drugs

There are five classes of antifungals: azoles, polyenes, pyrimidine analogues and echinocandins (Sanglard 2016). Azoles include fluconazole, itraconazole, and ketoconazole. This class of antifungal inhibits fungal ergosterol biosynthesis by inhibiting cytochrome P450 enzyme, 14-α sterol demethylase (Verweij et al. 2009). Polyenes include amphotericin B, which interacts with ergosterol in the fungal membrane, altering the membrane permeability. Pyrimidines like flucytosine inhibit DNA and RNA synthesis in pathogenic yeast and echinocandins, including caspofungin, anidulafungin, and micafungin, inhibit β 1-3 glucan synthase, which is essential in the biosynthesis of the fungal cell wall (Sanglard and Odds 2002). There is also a fifth class of antifungals known as allylamine antifungals, which include terbinafine. This antifungal works similarly to azoles in that it inhibits the ergosterol pathway, however, the target enzyme is squalene oxidase which leads to the accumulation of squalene and thus a deficiency of ergosterol in the fungal cell membrane (Vanreppelen et al., 2023). Of these antifungals, triazoles are the most prescribed treatment for invasive fungal infections (Vanreppelen et al., 2023; Sanglard 2016).

Sanglard (2016), along with other review articles (Chaabane et al., 2019; Krishnasamy et al., 2018) summarized the antifungal resistance mechanisms (Figure 2). Antifungal resistance mechanisms occur at a molecular level where alterations to genes that form part of the drug target may alter the susceptibility the cell has to the toxicity of the antifungal (Chaabane et al., 2019). These mechanisms can be divided into three major categories: decrease in effective drug concentration; alteration of drug targets and lastly metabolic bypass. A decrease in effective drug concentration can be achieved in three ways. Cells can develop an active efflux pump to remove antifungals taken up into the cells, drug targets can be overexpressed, or antifungal agents can be collected in intra- and extracellular compartments. Alteration of drug targets prevents antifungal drugs from inhibiting target enzymes as seen with azoles and echinocandins. Metabolic bypass can occur when there is a loss of or decrease in the specific function of a target metabolic pathway.

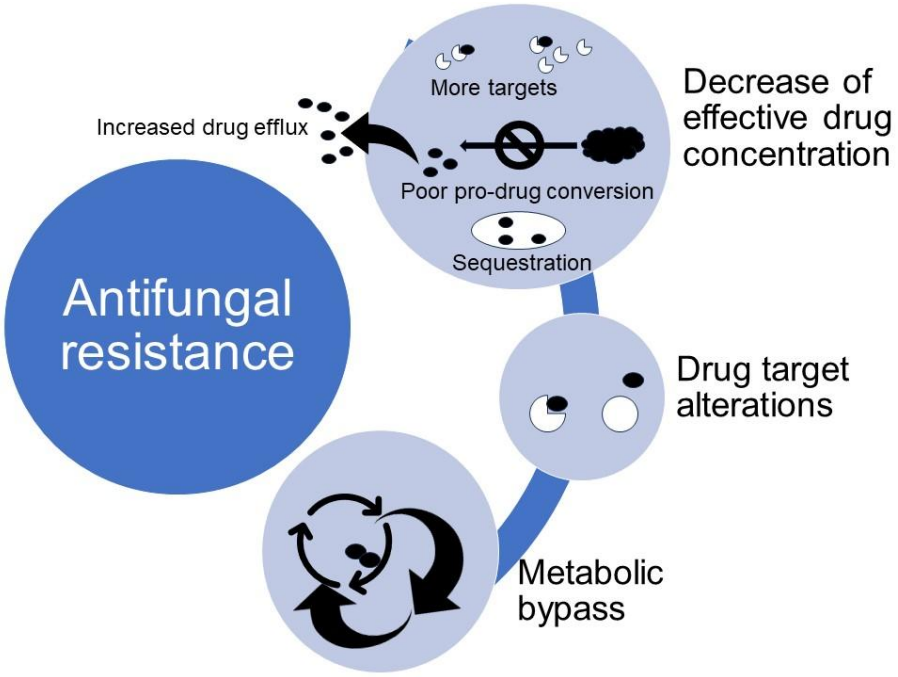


Figure 1 Illustration of the three main resistance mechanisms developed by fungi. 1) Decreased drug concentration efficiency, 2) Alterations to the drug targets and 3) Metabolic bypass of drug's target metabolic pathway

Antifungals in water

Wastewater treatment plants

Antifungals repeatedly found in WWTPs include clotrimazole, econazole, fluconazole, itraconazole, ketoconazole, posaconazole and miconazole (Assress et al., 2019; Assress et al., 2020; Huang et al., 2012; Peng et al., 2012; Kahle et al., 2008). Assress and co-workers (2019) found that fluconazole was present at the highest concentration of all five azoles identified in wastewater effluent. In a follow-up study, Assress and co-workers (2020) investigated the presence of azole antifungals in influent as well as effluent waters from three WWTPs and in a potable water treatment plant in South Africa. They identified six out of the eight target azole antifungal drugs to be present. It was also determined that fluconazole occurred at the highest concentration of up to 9.959 µg L^{-1}, this was followed by clotrimazole, ketoconazole and finally miconazole, econazole and itraconazole which were only found occasionally. Using statistical methods, the risk quotient was determined for various scenarios, including for drug resistance selection. This study determined that the fluconazole concentrations posed a moderate to high risk in all the studied treatment plants. Interestingly, fluconazole concentrations of influent and effluent wastewater are often similar, indicating the lack of effective treatment methods implemented at WWTPs (Peng et al., 2012; Kahle et al., 2008).

References

Arastehfar A, Carvalho A, Nguyen MH, Hedayati MT, Netea MG, Perlin DS, Hoenigl M (2020) COVID-19-Associated Candidiasis (CAC): An underestimated complication in the absence of immunological predispositions? J Fungi 6: 211. doi: https://doi.org/10.3390/jof6040211

Assress HA, Selvarajan R, Nyoni H, Ntushelo K, Mamba BB, Msagati TAM (2019) Diversity, co-occurrence and implications of fungal communities in wastewater treatment plants. Sci Rep 9: 14056. doi: https://doi.org/10.1038/s41598-019-50624-z

Assress HA, Nyoni H, Mamba BB, Msagati TAM (2020) Occurrence and risk assessment of azole antifungal drugs in water and wastewater. Ecotoxicol Environ Saf 187: 109868. doi: https://doi.org/10.1016/j.ecoenv.2019.109868

Amann R, Lemmer H, Wagner M (1998) Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques. FEMS Microbiol Ecol 25: 205-215. doi: https://doi.org/10.1111/j.1574-6941.1998.tb00473.x

Brumfield KD, Leddy M, Usmani M, Cotruvo JA, Tien CT, Dorsey S, Graubics K, Fanelli B, Zhou I, Registe N, Dadlani M (2022) Microbiome analysis for wastewater surveillance during COVID-19. Mbio 13: e00591-22. doi: https://doi.org/10.1128/mbio.00591-22

Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Yu T (2020) Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet 395: 507-513. doi: https://doi.org/10.1016/ S0140-6736(20)30211-7

Chaabane F, Graf A, Jequier L, Coste AT (2019) Review on antifungal resistance mechanisms in the emerging pathogen *Candida auris*. Front Microbiol 10: 2788. doi: https://doi.org/10.3389/fmicb.2019.02788

Cooke WB, Pipes WO (1969) The occurrence of fungi in activated sludge. Mycopathologia 40: 249–270. doi: https://doi.org/10.1007/BF02051779

Cooke WB (1970) Fungi associated with the activated-sludge process of sewage treatment at the Lebanon, Ohio, sewage treatment plant. O J Sci 70: 129–146.

Evans TN, Seviour RJ (2012) Estimating biodiversity of fungi in activated sludge communities using culture-independent methods. Microb Ecol 63: 773-86. doi: https://doi.org/10.1007/s00248-011-9984-7

Ezeokoli OT, Gcilitshana O, Pohl CH (2021) Risk factors for fungal co-infections in critically ill COVID-19 patients, with a focus on immunosuppressants. J Fungi 7: 545. Doi: https://doi.org/10.3390/jof7070545

Fakhru'l-Razi A, Molla AH (2007) Enhancement of bioseparation and dewaterability of domestic wastewater sludge by fungal treated dewatered sludge. J Hazar Mater 147: 350–356. doi: https://doi.org/10.1016/j.jhazmat.2007.01.060

Hart OE, Halden RU (2020) Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy, opportunities and challenges. Sci Total Environ 730: 138875. doi: https://doi.org/10.1016/j.scitotenv.2020.138875

Hoenigl M, Seidel D, Sprute R, Cunha C, Oliverio M, Goldman GH, Ibrahim AS, Carvalho A (2022) COVID-19-associated fungal infections. Nat Microbiol 7: 1127-1140. doi: https://doi.org/10.1038/s41564-022-01172-2

Huang Q, Zhang K, Wang Z, Wang C, Peng X (2012) Enantiomeric determination of azole antifungals in wastewater and sludge by liquid chromatography–tandem mass spectrometry. Anal Bioanal Chem 403: 1751–1760. doi: https://doi.org/10.1007/s00216-012-5976-9

Janahi EM, Mustafa S, Parkar SF, Naser HA, Eisa ZM (2020) Detection of enteric viruses and bacterial indicators in a sewage treatment center and shallow water bay. Int J Environl Res Public Health 17: 6483. doi*:* https://doi.org/10.3390/ijerph17186483

Kacprzak M, Neczaj E, Okoniewska E (2005) The comparative mycological analysis of wastewater and sewage sludges from selected wastewater treatment plants. Desalination 185: 363–370. doi: https://doi.org/10.1016/j.desal.2005.03.085

Kahle M, Buerge IJ, Hauser A, Muller MD, Poiger T (2008) Azole fungicides: occurrence and fate in wastewater and surface waters. Environ Sci Technol 42: 7193-7200. doi: https://doi.org/10.1021/es8009309

Krishnasamy L, Krishnakumar S, Kumaramanickavel G, Saikumar C (2018) Molecular mechanisms of antifungal drug resistance in *Candida* species*.* J Clin Diag Res 12: DE01-DE06. doi: 10.7860/JCDR/2018/36218.11961

McCall C, Wu H, Miyani B, Xagoraraki I (2020) Identification of multiple potential viral diseases in a large urban center using wastewater surveillance. Water Res 184: 116160. doi: https://doi.org/10.1016/j.watres.2020.116160

More TT, Yan S, Tyagi RD, Surampalli RY (2010) Potential use of filamentous fungi for wastewater sludge treatment. Bioresour Technol 101: 7691-7700. doi: https://doi.org/10.1016/j.biortech.2010.05.033

Moser D, Biere K, Han B, Hoerl M, Schelling G, Choukér A, Woehrle T (2021) COVID-19 impairs immune response to *Candida albicans*. Front Immunol 12: 640644. doi: https://doi.org/10.3389/fimmu.2021.640644

Paul JR, Trask JD, Culotta CS (1939) Poliomyelitic virus in sewage. Science 90: 258-259.

Peng X, Huang Q, Zhang K, Yu Y, Wang Z, Wang C (2012) Distribution, behavior and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci Total Environ 426: 311-7. doi: https://doi.org/10.1016/j.scitotenv.2012.03.067

Sanglard D (2016) Emerging threats in antifungal-resistant fungal pathogens. Front Med 3: 11. doi: https://doi.org/10.3389/fmed.2016.00011

Sanglard D, Odds FC (2002) Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis 12: 73-85. doi: https://doi.org/10.1016/S1473-3099(02)00181-0

Seagle EE, Jackson BR, Lockhart SR, Georgacopoulos O, Nunnally NS, Roland J, Barter DM, Johnston HL, Czaja CA, Kayalioglu H, Clogher P (2022) The landscape of candidemia during the coronavirus disease 2019 (COVID-19) pandemic. Clin Infect Dis 74: 802-811. doi: https://doi.org/10.1093/cid/ciab562

Song G, Liang G, Liu W (2020) Fungal co-infections associated with global COVID-19 pandemic: A clinical and diagnostic perspective from China. Mycopathologia 185: 599–606. doi: https://doi.org/10.1007/s11046-020-00462-9

Vanreppelen G, Wuyts J, Van Dijck P, Vandecruys P (2023) Sources of antifungal drugs. J Fungi 9: 171. doi: https://doi.org/10.3390/jof9020171

Verweij PE, Snelders E, Kema GHJ, Mellado E, Melchers WJG (2009) Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis 9: 789–795. doi: https://doi.org/10.1016/S1473-3099(09)70265-8

Yang Q, Angly FE, Wang Z, Zhang H (2011) Wastewater treatment systems harbour specific and diverse yeast communities. Biochem Eng J 58– 59: 168– 176. doi: https://doi.org/10.1016/j.bej.2011.09.012

Yang Q, Zhang H, Li X, Wang Z, Xu Y, Ren S, Chen X, Xu Y, Hao H, Wang H (2013) Extracellular enzyme production and phylogenetic distribution of yeasts in wastewater treatment systems. Bioresour Technol 129: 264-273. doi: https://doi.org/10.1016/j.biortech.2012.11.101

Chapter 2:

Optimisation of experimental procedures on wastewater samples

Abstract

Wastewater samples prove to be complex samples producing various challenges during downstream analysis. Wastewater is riddled with PCR inhibitors that hinder the effective functioning of DNA-polymerases during PCR, these inhibitors need to be efficiently removed which can be achieved through various methods. Another major challenge presented is the low concentration genetic material present in wastewater samples. Thus, optimisation is essential when it comes to analysis of these samples. Through the experimental work performed in this chapter, it was determined that the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit was most efficient for the extraction of genomic DNA and that filtration of samples through a 0.45 µm nitrocellulose filter disc along with centrifugation are efficient in concentrating enough cells for DNA extractions. The effectiveness of ethidium bromide monoazide in the amplification of only viable cells' DNA was also investigated, and proved to be efficient at a final concentration of 6 µM. Three different types of polymerases were also tested for use in the multiplex PCR where the Q5 DNA polymerases yielded the best results. The optimised methods determined in this chapter can support further work on validating wastewater surveillance of pathogenic yeast.

Keywords: Ethidium monoazide bromide, High-fidelity DNA-Polymerase, Multiplex PCR

Introduction

The emerging threat of fungal infections is frequently overlooked, with over 150 million people having serious fungal infections that range from asymptomatic to fatal outcomes, annually (Sharma and Chakrabart, 2023; Bongomin et al. 2017). When comparing the mortality associated with fungal infections to the mortality associated with other diseases, such as tuberculosis (TB) and malaria, fungal infections prove to be very significant, with slightly higher associated mortality than TB and almost three times that of malaria (Kainz et al., 2020; Bongomin et al. 2017). Candidiasis is the most common form of fungal infection and is caused by species of the *Candida* genus (Sharma and. Chakrabart, 2023; Parslow and Thornton 2022). Other fungal species causing serious fungal infections belong to the genera *Aspergillus, Pneumocystis* and *Cryptococcus* (Rajasingham et al. 2022; Fishman, 2020; Bongomin et al. 2017; Kosmidis and Denning, 2015). The world Health Organisation (WHO) recently published a list of priority fungal pathogens, focusing on fungal agents responsible for systemic infections, in which various fungi were classified into critical-, high-, and medium-priority groups (WHO, 2022). Within these groups, *Candida, Cryptococcus* and *Aspergillus* species are classified into the critical group with numerous other *Candida* spp. appearing in the high and medium groups. This list further supports the urgent need to investigate these fungal pathogens along with the development of efficient treatment protocols and diagnostic tests to prevent fatal outcomes.

The global disease burden of fungal infection is only partially understood due to a lack of data from developing countries (Bongomin et al. 2017, Denning 2017). The lack of data may be attributed to various factors such as a lack of published research and national surveillance systems as well as limitations regarding methodology and diagnostic tests (Pegorie et al., 2017). An array of diagnostic tests is available, each with different levels of sensitivity, specificity, limitations and advantages. Diagnostic tests can be divided into non-molecular and molecular tests. Non-molecular methods include culturing, and staining followed by histopathology of samples which may be swabs, tissue- or blood- samples (Arvanitis et al., 2014; Stevens, 2002) the use of a β-glucan assay may also prove useful where there is a decrease in the sensitivity of culturing. The β-glucan test detects the 1,3-β-D-glucan polysaccharide component of the fungal cell wall which will be present in the blood serum of patients infected by fungal agents (Hachem et al., 2009; Kedzierska et al., 2007, Stevens, 2002). Serological tests which detect specific antigens presented by fungal microorganisms using the appropriate antibodies may also be used (Moragues et al., 2004). Non-molecular tests are well-developed and frequently used, but lack optimal sensitivity (Racil et al., 2010), are often time-consuming (Arvanitis et al., 2014;

Rajkumari et al., 2014) and need to be performed in conjunction with other diagnostic tests (Hachem et al., 2009) so the need arises to develop tests that are both time efficient and highly sensitive.

The use of molecular methods for identification of fungal pathogens are advantageous, due to their improved sensitivity and specificity, and rapid turnover time (Arvanitis et al., 2014; Stevens, 2002). Polymerase chain reaction (PCR) is frequently used to identify pathogens using primers that amplify a target region of DNA. There are other molecular tests available which were designed to either improve on the conventional PCR or to be used separately, these include multiplex PCR (Aboutalebian et al., 2021), fluorescence *in situ* hybridization (FISH) (Da Silva et al., 2015), nucleic acid sequence-based amplification (NASBA) (Widjojoatmodjo et al., 1999), MALDI-TOF MS (Patel, 2015), and other novel tests based on a spectroscopic approach as well as the use of microscopic resonating cantilevers (Arvanitis et al., 2014; Nugaeva et al., 2005).

Focusing on the multiplex PCR, this technique enables the amplification of multiple target DNA sequences simultaneously in one reaction, using multiple primers and a thermostable DNA polymerase (Mahoney et al., 2007; Persson et al., 2005; Elnifro et al., 2000) as depicted in figure 2.1. This type of PCR was designed to be time efficient (Elnifro et al., 2000; Persson et al., 2005; Birch, 1996) and less labour-intensive than conventional PCR or diagnostic tests (Aboutalebian et al., 2021). This technique has been previously used in diagnostic tests that target viruses, bacteria, parasites and fungi (Aboutalebian et al., 2021; Monila et al., 2015; Taniuchi et al., 2011; Mahoney et al., 2007; Cassinotti and Siegl, 1998). Thus, it shows promise as a rapid and reliable test that may be routinely performed in diagnostic laboratories. The challenge associated with multiplex PCR is that it requires stringent optimisation, especially when it comes to the primer sets used (Ali et al., 2014; Persson et al., 2005; Elnifro et al., 2000). The primers must be designed to ensure that all the primer sets can efficiently anneal to the template at the same temperature (universal conditions) along with preventing the formation of primer dimers and secondary structures in the primers (Ali et al., 2014; Birch, 1996).

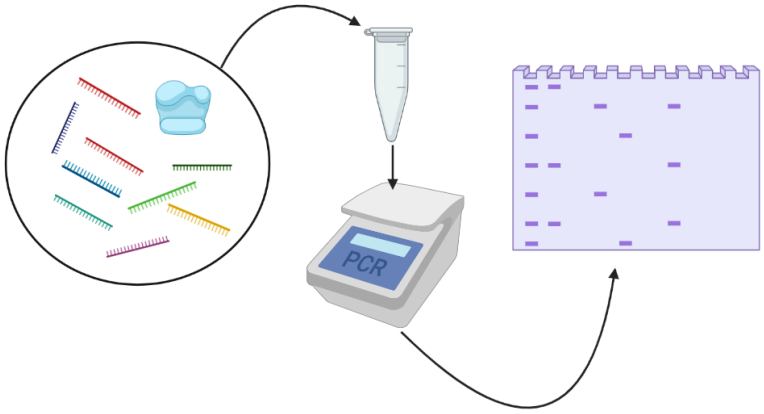


Figure 2.1 Diagram of the basic workflow of a multiplex PCR. Multiples primer pairs are used along with a suitable thermostable DNA-Polymerase in one reaction to amplify DNA sequences of multiple microorganisms present in one sample. The resulting amplicons can then be separated visualised using gel electrophoresis

This chapter will focus the optimisation of culture dependent methods (i.e., isolation) of yeasts, as well as a multiplex PCR to detect multiple fungal pathogens in wastewater samples. This optimization will be performed using numerous DNA extraction kits to determine which will be most efficient in extracting DNA from the complex matrix that is wastewater, as well as two different DNA polymerases and the use of a PCR pre-mix to amplify the DNA sequences of target pathogens for efficiently and accurate identification.

Materials and Methods

Yeast Isolation

Various methods were investigated to isolate yeast from wastewater. The first method was to filter 50 ml of wastewater, as is, the second method was to centrifuge 50 ml of wastewater at 7830 rpm (Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor) and then filter the supernatant. Lastly, the pellet that remained after centrifugation was resuspended in phosphate saline buffer (PBS) (Basingstoke, Hampshire, United Kingdom) and filtered. All three attempts were filtered using a 0.45 µm cellulose nitrate filter (Satorius; Germany). Filter disks were then incubated on sabouraud dextrose agar (SDA) supplemented with chloramphenicol (10 g/L peptone powder, 40 g/L dextrose, 500 mg/L chloramphenicol, 15g/L bacteriological agar - Sigma Aldrich; Missouri USA) at 37°C for 48 hours. Randomly selected yeast colonies were confirmed to be yeast cells using light microscopy.

Genomic DNA extraction

Wastewater samples were collected, and genomic DNA was extracted using various extraction kits. These kits were: Norgen Biotek Corp Fungi/Yeast Genomic DNA Isolation Kit (Thorold, Ontario, Canada); ZYMO research Quick DNA™ Fungal/Bacterial Miniprep Kit (Orange; California); ZYMO research Quick DNA™ Fecal/Soil Microbe Miniprep DNA Kit (Orange; California); OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit (Georgia; USA). For the first two kits a volume of 50 ml of wastewater was used, and the extraction was performed according to the manufacturer's protocol. The 50 ml wastewater sample was centrifuged at 7830 rpm (Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor), and the supernatant, as well as the resulting pellet (resuspended in PBS), was used separately to extract genomic DNA from. The final elution step was also adjusted to use 60 µl elution buffer. For the ZYMO research Quick DNA™ Fecal/Soil Microbe Miniprep DNA Kit and the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit, 50 ml of a wastewater sample was filtered using a 0.45 µm cellulose nitrate filter paper (Satorius, Germany). The filter papers were then used for DNA extraction according to the manufacturer's protocol. Column equilibration for the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit was done according to the manufacturer's protocol and the column wash step was done once. The final elution step for both kits were also adjusted to use 60 µl elution buffer. In all cases the eluted DNA was visualised using gel electrophoresis (0.8% agarose gel at 90V, 400 mA for 25 minutes). GeneRuler DNA Ladder Mix, ready-to-use (ThermoFischer Scientific, Walthan USA) was used as a marker.

Multiplex Polymerase chain reaction (PCR)

Three multiplex reactions were set up containing various yeast of interest. Primer design was done according to an article by Arastehfar et al. (2019) and ordered from Integrated DNA Technologies (Iowa USA). Primer sequences are listed in Table S1 in the supplementary data. Yeast isolates for each multiplex reaction were obtained from SANBI Biobank SA Yeast culture collection at the Department of Microbiology and Biochemistry, University of the Free State. All isolates were cultured in yeast malt broth (peptone powder 5g/L, malt extract 3g/L, yeast extract 3g/L and glucose 10g/L- Sigma Aldrich; Missouri USA) at 37°C for 24 hours.

Multiplex one included the following important pathogenic *Candida* species: *Candida albicans, C. auris*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *Pichia kudriavzevii* (anamorph = *Candida krusei*). Genomic DNA from liquid pure cultures of these isolates as well as a mixed sample, containing one ml of each of the individual cultures, was extracted using the Zymo Research Quick-DNA™ Bacterial/ Fungal miniprep kit (Orange; California).

Multiplex two included the following minor pathogenic yeast species: *Meyerozyma guilliermondii* (anamorph = *Candida guilliermondii*), *Kluyveromyces marxianus, Clavispora lusitaniae* (anamorph = *Candida lusitaniae*)*, Pichia norvegensis* (anamorph = *Candida norvegensis), Debaromyces hansenii, Yarrowia lipolytica* and *Diutina rugosa* (anamorph = *Candida rugosa*), while multiplex three included *Cryptococcus neoformans*, *Cryptococcus gattii*, *Rhodotorula mucliginosa* and *Trichosporon asahii.* Genomic DNA from liquid cultures of these isolates, as well as a mixed sample of each multiplex was extracted using the Omega Bio-Tek E.N.Z.A. ® Water DNA kit (Georgia; USA).

In all cases, the presence of genomic DNA was confirmed using gel electrophoresis (0.8% agarose gel at 90 V, 400 mA for 25 minutes). GeneRuler DNA Ladder Mix, ready-to-use (ThermoFischer Scientific, Walthan USA) was used as a marker.

PCR amplification

PCR was done using the Applied biosystems 2720 Thermocycler (Applied Biosystems, Massachusetts USA) using different PCR systems.

For the KAPA Taq PCR kit (KAPA Biosystems; Massachusetts USA), the reaction volume for individual yeast isolates was a final total of 25 µl and the reaction composition was as follows: 5 µl of a 10X KAPA Taq buffer A, 1 µl of a 2 mM dNTP mix, 1 µl [10 µM] forward primer (0.4 µM final concentration), 1 µl [10 µM] reverse primer (0.4 µM final concentration), 1 µl DNA template, 0.2 µl of Taq (0.2 U final concentration) and 16 µl molecular grade water. The same reaction was made up in a second attempt using a 5 µl DNA template, 1 µl of a 10mM dNTP mix and 11 µl molecular grade water.

The reaction for the mixed samples was made up to a total final volume of 50 µl: 5 µl of a 10X KAPA Taq buffer A, 1 µl of a 2 mM dNTP mix, 1 µl [10 µM] forward primer (0.2 µM final concentration), 1 µl [10 µM] reverse primer (0.2 µM final concentration), 1 µl DNA template, 0.2 µl of Taq (0.2 U final concentration) and 29 µl molecular grade water. The same reaction was made up in a second attempt using 5 µl of DNA template, 1 µl 10mM dNTP mix and 25 µl molecular grade water.

PCR conditions were as follows: initial denaturation at 95°C for 5 minutes (1 cycle), denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute (each for 35 cycles), and lastly final extension at 72°C for 8 minutes. This was used for the first attempt. In the second attempt (5 µl DNA template) the annealing temperature was lowered to 50°C.

For the AccuStart II PCR ToughMix® (2x) (Quantabio, Massachusetts; USA), reactions for individual yeast isolates were made up to a final volume of 25 µl: 12.5 µl AccuStart II PCR ToughMix® (2X), 1 µl of

a 10 mM dNTP mix, 1 µl [10 µM] forward primer (0.4 µM final concentration), 1 µl [10 µM] reverse primer (0.4 µM final concentration), 5 µl DNA template and 5.5 µl molecular grade water. The same reaction was made up in a second attempt using an additional 25 mM $MgCl_2$ reagent at 3,4- and 6-mM concentrations respectively. The reaction for the mixed samples was made up to a volume of 50 µl: 25 µl of AccuStart II PCR ToughMix® (2X), 1 µl [10 µM] forward primer (0.2 µM final concentration), 1 µl [10 µM] reverse primer (0.2 µM final concentration), 5 µl DNA template, and 6 µl molecular grade water.

PCR conditions were as follows: initial denaturation at 95°C for 5 minutes (1 cycle), denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minute (each for 35 cycles), and lastly final extension at 72°C for 8 minutes.

For the Q5® High-Fidelity DNA Polymerase (New England Biolabs; Massachusetts USA), the reaction mixture for individual yeast samples was made up to a final volume of 25 µl and contained: 5 µl of a (5x) reaction buffer, 0.5 µl of 10mM dNTPs, 0.5 µl [20 µM] forward primer (0.4 µM as final concentration), 0.5 µl [20 µM] reverse primer (0.4 µM as final concentration), 5 µl DNA template, 0.25 µl Q5® high fidelity polymerase and 13.5 µl Molecular grade water. The same reaction was made up again using 1 µl [20 µM] of the forward and reverse primers (final concentration 0f 0.2 µM) and 112,5 µl molecular grade water.

The reaction mixture for the mixed samples was made up to a final volume of 50 µl and contained: 10 µl of a (5x) reaction buffer, 1 µl of 10mM dNTPs, 0.5 µl [20 µM] forward primer (0.4 µM as final concentration), 0.5 µl [20 µM] reverse primer (0.4 µM as final concentration), 5 µl DNA template, 0.5 µl Q5® high fidelity polymerase and 26.5 µl Molecular grade water. The same reaction was made up using 1 µl [20 µM] of each forward and reverse primer and 19.5 µl molecular grade water.

PCR conditions were as follows: initial denaturation at 95°C for 5 minutes (1 cycle), denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 40 seconds (each for 35 cycles), and lastly final extension at 72°C for 8 minutes.

In all cases, PCR amplicons were visualised using gel electrophoresis (0.8% agarose gel at 90V, 400mA for 30 minutes). GeneRuler DNA Ladder Mix, ready-to-use (ThermoFischer Scientific, Walthan USA) was used as a marker.

Multiplex PCR on wastewater sample

The genomic DNA extracted from wastewater using the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit (Georgia; USA) was used as a template in the reaction, using Q5® High-Fidelity DNA Polymerase.

The multiplex reaction contained the following species-specific primers: multiplex 1 contained the primers for *C. albicans, C. auris*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *P. kudriavzevii.* Multiplex 2 was divided into two individual reactions, multiplex 2a (*M. guiliermondii*, *K. marxianus, C. lusitaniae,*) and multiplex 2b (*D. hansenii, Y. lipolytica, P. norvegensis* and *D. rugosa)*. Multiplex 3 contained primers for *C. neoformans*, *C. gattii*, *R. mucliginosa* and *T. asahii.*

The effect of ethidium bromide monoazide (EMA) was also determined (Reyneke et al., 2017). A 5mg/ml stock of EMA (Invitrogen-Thermo Fisher Scientific, Massachusetts USA), dissolved in a 20% dimethyl sulfoxide (Sigma Life Science, Missouri USA), was added to the 50 ml wastewater sample to a final concentration of 6 µM. Samples were vortexed and incubated horizontally on ice for 10 minutes in the absence of light. Incubation was followed by a 15-minute exposure to a halogen light source. Samples were washed with 1 ml 0.85% NaCl solution and centrifuged at 7830 rpm (Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor) after which the supernatant was discarded, and the remaining pellet was resuspended in 30 ml PBS.

Further validation of multiplex PCR accuracy

Mapping of primers to target sequences

Primers and target sequences for each multiplex set were obtained from the supplementary material of the article by Arastehfar and colleagues (2019) and were mapped using the following website https://www.genecorner.ugent.be/primer_map.html. The previously mentioned websites identified the annealing position of primers to sequences submitted to the website.

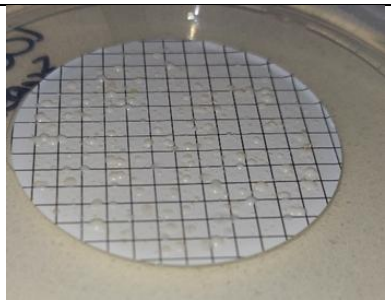
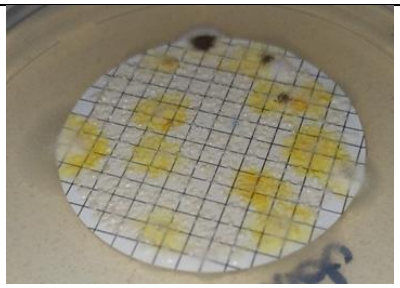
Results and Discussion

Yeast isolation

From the three methods used to isolate yeasts from wastewater, the second attempt using only the supernatant of the sample, yielded the best results. In contrast to all the other methods, no molds were isolated that may hinder the effective enumeration and isolation of yeast colonies (Table 2.1). The six colonies selected from this plate using the Harrison disc method all showed the characteristic

size and morphology expected from yeast cells. This included an ovoid shape, a size of about 3 to 5 µm in diameter as well as the formation of budding cells.

Table 2.1 Results from different methods of yeast isolation on cultivated on sabouraud dextrose agar

Sample	Outcome	Image
1. 50 ml of wastewater, not centrifuged	Visible yeast colonies but overgrown with mold	
2. 50 ml of wastewater supernatant	Visible yeast colonies, no mold growth	
3. 50 ml of resuspended wastewater pellet	Visible yeast colonies, with mold growth	

All three filter discs had abundant growth due to the nutrient rich environment presented by wastewater. Wastewater is typically rich in dissolved organic and inorganic matter which includes a complex mixture of monomers such as monosaccharides, amino acids, fatty acids as well as their polymeric counter parts, other matter present include humic substances and pollutants due to human activity (Shi et al., 2021). Pollutants include heavy metals, pharmaceutical products as well as other endocrine disrupting compounds (Huang et al. 2010; Lindberg et al. 2010; Jones et al., 2005; Qdais et al., 2004). This collection of matter can dramatically influence the level of dissolved oxygen present in wastewater and thus the composition of microorganisms present, which may include pathogens as well as non-pathogenic strains of bacteria and fungi (Nicula et al., 2023).

The identification of yeast in wastewater can be done through culture-dependent and independent techniques. Culture-dependent techniques are often time-consuming (McLain et al., 2016), labour-intensive and show a biased overview of microbial presence since the artificial media used for culturing only allows the growth of a select proportion of microorganisms (Carraro et al., 2011) and excludes the so-called non-culturable microorganisms. Culture-independent techniques, or molecular techniques, on the other hand are time and labour efficient and has the added advantage of being able to distinguish between closely related strains which by any morphological observation would be seen as the same species. Yet the advantages of culture-dependent techniques must not be overlooked. When it comes to the investigation of the resistance of isolates to antimicrobials, the ability to culture and determine the level of resistance or susceptibility of the isolates through culturing provides valuable data which might not have been obtained by solely looking into the isolate's genotype. The data yielded from both culture-dependent and -independent techniques should be used in conjunction to obtain accurate and complete insight into the traits and abilities of different microbial species.

Numerous studies investigating the presence of fungi in water or wastewater make use of chloramphenicol supplementation in media to prevent the growth of various unwanted bacterial species (Caicedo-Bejarano et al., 2023; Novak Babič et al., 2016; Medeiros et al., 2012; Pereira et al., 2009). Chloramphenicol is a broad-spectrum antibiotic used in the veterinary as well as medical fields (Dinos et al., 2016; Pongs, 1979). This antibiotic specifically targets the large ribosomal (50S) subunit of the 70S bacterial ribosome, and its mechanism entails the inhibition of peptidyl transferase activity thus preventing protein formation (Choi et al., 2020; Dinos et al., 2016). This makes this antibiotic ideal for the selection of fungi growth on the selected medium from samples such as wastewater that contain a diverse community of various microorganisms.

The successful isolation of yeast strains from wastewater and their subsequent identification, which will be discussed in the following chapter, together with the data obtained from multiplex PCR will highlight the overall yeast diversity found in this environment as well as indicate which pathogenic yeast strains are present.

Genomic DNA extraction

From figure 2.2 DNA was extracted from the pellet of a sample using the Norgen kit as well as Zymo fecal/Soil kit. The latter kit was also able to extract DNA from the supernatant that had been filtered. The Omega kit also performed well; this kit also instructs the user to filter samples through a filter

paper. In both the Zymo fecal/Soil kit and the Omega kit the filter paper, after the samples had been filtered, was used as a starting point for the extraction protocol.

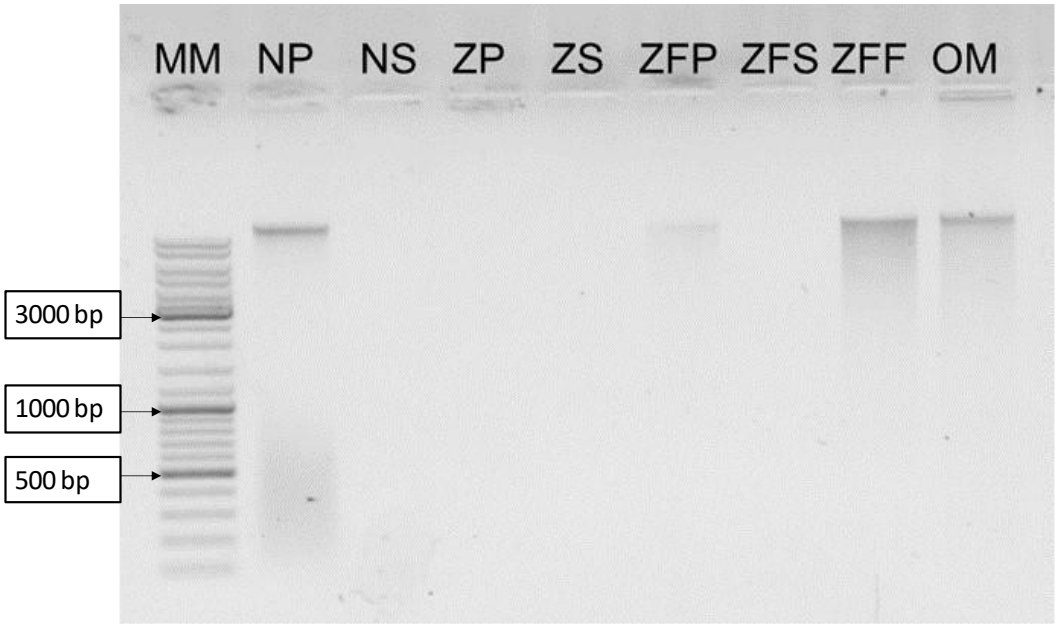


Figure 2.2 Gel electrophoresis images of genomic DNA extraction using various kits. N represents the Norgen Bio-Tek Kit and Z the Zymo research quick DNA^{TM} Fungal/Bacterial kit. ZF represents the Zymo research Quick DNA^{TM} Fecal/Soil Microbe Miniprep DNA Kit and OM represents the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit. In both images the P represents the use of the sample pellet for DNA extraction and S the use of sample supernatant. F refers to the use of a filter paper for extraction.

A major challenge associated with wastewater surveillance is the complexity of the wastewater matrix (Ahmed et al., 2022; Kumblathan et al., 2022; Ahmed et al., 2020), which may contain PCR inhibitors that hinder downstream applications and thus needs to be removed to ensure efficient and accurate analysis. These PCR inhibitors include proteins, carbohydrates, fats, metal ions, RNase, polyphenols as well as other organic and inorganic substances that may dissolve in the water or remain solid (Sidstedt et al., 2020; Acharya et al., 2017; Schrader et al., 2012). Methods used to circumvent this challenge is to either remove the inhibitors during the extraction of genetic material or to use an inhibitor resistant DNA polymerase during downstream PCR or qPCR applications (Ahmed et al., 2022). It is important to considered which PCR inhibitors are present in samples to select the most suitable methods for the removal of these inhibitors. Wastewater samples, for example, are a goldmine for inhibitors

considering all the types of waste collected in wastewater transport networks, a few of these inhibitors include urea (mainly from urine), polysaccharides, bile acids, chlorophyll and an array of different medications (Schrader et al., 2012).

Another general concern regarding wastewater surveillance studies is the effective extraction of often low-concentration genetic material from samples (Juel et al., 2021; Ahamed et al., 2020; Farkas et al., 2018). Thus, different method for the concentration of samples and composition of the spin column membranes used during nucleic acid extraction needs to be considered. This concern will be addressed in this discussion.

Considering how diluted the wastewater samples are, the efficient analyses of wastewater samples will only be achieved by concentrating the genetic material present. Different concentration methods include various forms of filtration (electro-positive or -negative and tangible ultrafiltration), ultracentrifuge-based methods and skimmed milk flocculation (Juel et al., 2021; Farkas et al., 2018). Most of these methods have been applied to the concentration of viruses or viral particles found in wastewater, but similar principles apply to the isolation of yeast from wastewater. The use of a membrane filter with a pore size of 0.45 µm to isolate yeast from various water sources is widely reported in literature (Babler et al., 2023; Arroyo et al., 2020; Novak Babič et al., 2016; van Wyk et al., 2012; Pereira et al., 2009). Thus, it's not surprising that the extraction attempts using filter discs (as seen in lanes ZFF and OM) performed better than the attempts on the sample supernatant and pellet (as seen in lanes ZP, ZS, ZFP and ZFS).

The type of membrane used in the spin columns play an essential role in the efficient extraction of pure nucleic acids or plasmids (Yang et al., 2008). During optimization, different commercially available DNA extraction kits were tested on wastewater samples. The Norgen Bio-Tek Kit contains an ion exchange resin while the Zymo research quick DNA™ Fungal/Bacterial kit, Zymo research Quick DNA™ Fecal/Soil Microbe Miniprep DNA Kit the OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit makes use of a silica bead-based membrane. Ion exchange membranes hold an advantage over silica-based membranes due to its higher affinity towards larger biomolecules such as genomic DNA and plasmid DNA and lower affinity towards small biomolecules which may include different impurities and small proteins (Yang et al., 2008). Silica-based membranes on the other hand have a greater binding capacity for small molecules due to the larger target biomolecules only binding to the available bead surface which allows smaller molecules to bind in the smaller spaces left between beads (Endres et al., 2003). This may be the reason why the Norgen kit worked and the Zymo fungal/bacterial kit did not.

The reasons for the difference in performance between the two Zymo research kits are still unclear (comparing lanes ZP, ZS to ZFP and ZFS respectively) since both follow the same protocol and use the same reagents, yet the Zymo Quick DNA™ Fecal/Soil Microbe Miniprep DNA Kit did yield genetic material from the sample pellet whereas the Zymo fungal/bacterial kit did not. However, the Zymo research Quick DNA™ Fecal/Soil Microbe Miniprep DNA Kit contains an additional purification step using a column that specialises in the removal of PCR inhibitors like polyphenols and organic acids but is only used at the end of the protocol making it unclear why there was the observed differences. The OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit makes use of a filtration step, isopropanol precipitation and a silica-based membrane spin column which all might contribute to the efficiency of this kit for the extraction of genomic DNA from wastewater samples.

Validation of multiplex PCR of laboratory cultures

The multiplex PCR was tested on the group of yeasts that are listed under multiplex one in Table S1 using Kapa Taq. As indicated in figure 2.3a, only the pure culture of *C. tropicalis* showed an expected band size of 126 base pairs (bps), while the mixture of all the yeast (Mx1) showed four bands out of the expected seven. From top to bottom, these bands were approximately 700 bps (*C. dubliniensis*), 400 bps (*C. auris*), 200 bps (*C. glabrata*) and 126 bps (*C. tropicalis*). This was repeated with a higher concentration of dNTPs and DNA template as well as a lower annealing temperature (figure 2.3b). However, only *C. glabrata* and *C. tropicalis* showed expected band sizes of approximately 200 bps and 126 bps, while the multiplex did not show any of the seven expected bands. In both figures, the failed PCRs had prominent primer dimers or non-specific binding products collecting at the bottom of the gel.

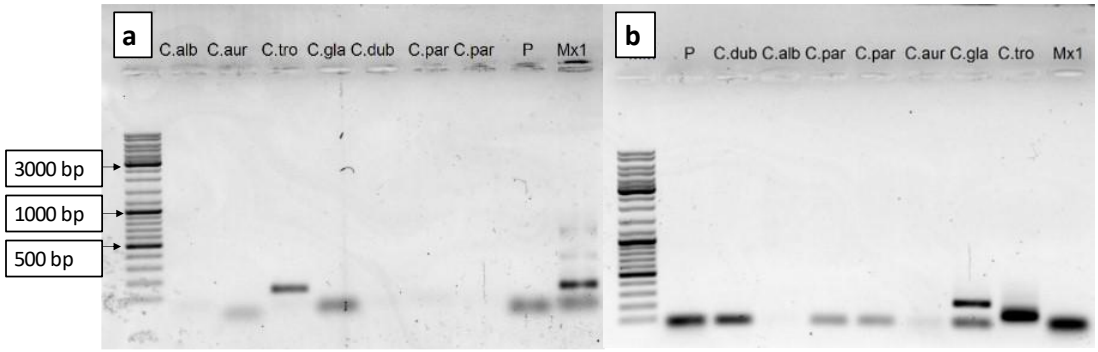


Figure2.3 PCR using Kapa Taq for pure cultures and multiplex 1. 2.3a) PCR done with 2 mM concentration of a dNTP mixture, 60°C annealing temperature and 1 µl DNA template. 2.3b) PCR done with a 10mM concentration of a dNTP mixture, 50°C annealing temperature and 5 µl DNA template. C.alb (*C. albicans*), C.aur (*C. auris*), C.tro (*C. tropicalis*), C.gla (*C. glabrata*), C.dub (*C. dubliniensis*), C.par (*C. parapsilosis*), P (*Pichia kudriavzevii*) and Mx1 (mixed sample)

The same yeasts were used in a PCR using AccuStart II PCR ToughMix® (2x) (Figure 2.4). The expected band were observed for the individual yeasts. However, in the multiplex reaction, three out of the seven expected bands were seen. These bands present were the size expected for *C. albicans* (600 bps)*, C. glabrata* (200 bps) and *C. troplicalis* (126 bps).

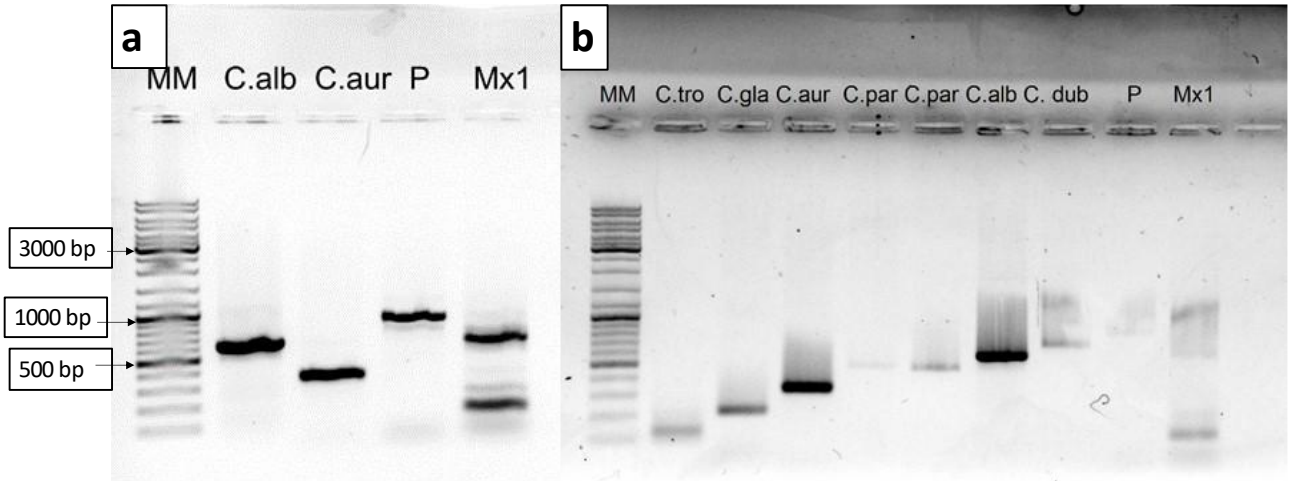


Figure 2.4 PCR using AccuStart II PCR ToughMix® (2x) pre-mix for pure cultures and multiplex 1. 2.4a) initial trail run on selected isolates as well as the sample containing genomic DNA from all the isolates. 2.4b) complete PCR on all the isolates of multiplex 1 as well as the mixed sample. C.alb (*C. albicans*), C.aur (*C. auris*), C.tro (*C. tropicalis*), C.gla (*C. glabrata*), C.dub (*C. dubliniensis*), C.par (*C. parapsilosis*), P (*Pichia kudriavzevii*) and Mx1 (mixed sample)

The addition of $MgCl_2$ to the samples containing a mixture of isolates from multiplex one did not yield any of the expected band sizes. Primer dimers or non-specific binding products did however form at the bottom of the gel (Figure 2.5).

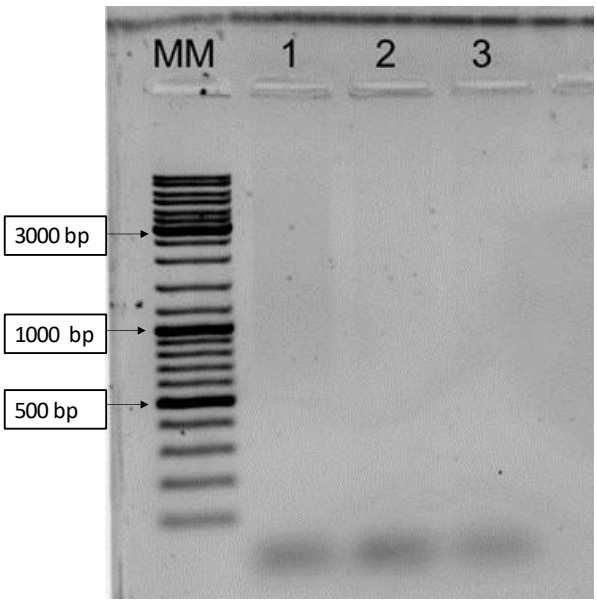


Figure 2.5 PCR using AccuStart II PCR ToughMix® (2x) and various $MgCl_2$ concentrations on multiplex 1. Lane 1,2 and 3 contains 3 mM, 4 mM and 6 mM $MgCl_2$ respectively

Using Q5® High-Fidelity DNA Polymerase, PCR and a multiplex PCR was performed on the group of yeasts (figure 2.6) that are listed under multiplex 2a and b in Table S1. Lane 1 contains a control of *K. marxianus* which did show an expected band size of approximately 200 bps. Lanes 2 and 3 contain the genetic material of samples with a mixture of yeast isolates that are listed under multiplex two (Mx2). Lane 2 contains primers at a final concentration of 0.2 µM and lane 3 contains primers at a final concentration of 0.4 µM. In both lane 2 and 3, 4 out of 7 expected bands were seen clearly. The banding pattern, from top to bottom, represent *P. norvegensis* (500 bps), *C. lusitiniae* (400 bps), *M. guilliermondii* (300 bps) and *K. marxianus* (200 bps).

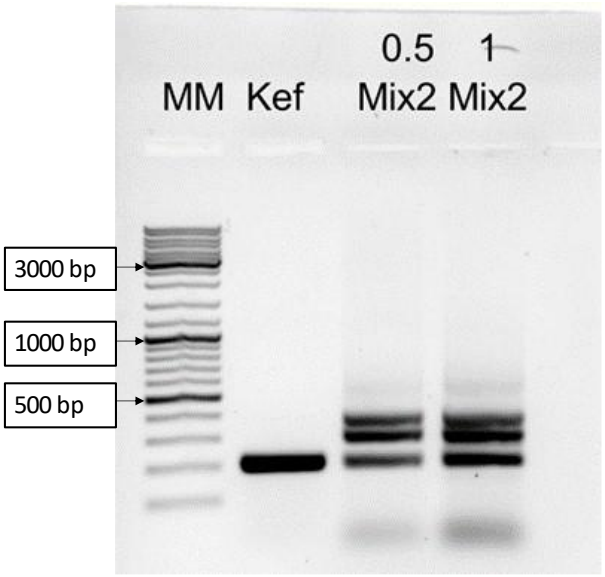


Figure 2.6 PCR using Q5® polymerase on a control and multiplex 2. The lane marked 0.5 containes primers at a final concentration of 0.2 µM and lane 1 contains primers at a final concentration of 0.4 µM. Kef (*K. marxianus*)

In figure 2.7a the PCR using Q5 polymerase worked on all the individual isolates from multiplex one as well as the mixed sample, were 6 out of the 7 expected bands were seen. In the Mixed sample (Mx1) the banding pattern, from top to bottom, represents *C. dubliniensis* (700 bps), *C. albicans* (600 bps), *C. parapsilosis* (500 bps), *C. auris* (400 bps), *C. glabrata* (200 bps) and *C. tropicalis* (126 bps). The only band not present is that of *Pichia kudriavzevii*.

In figure 2.7b the PCR worked on all the individual isolates from multiplex 2 except *Pichia norvegensis*. The multiplex reaction for the mixed samples were split into multiplex 2a and b (Mx2a, Mx2b). The banding pattern for Mx2a, from top to bottom, represents *Clavispora lusitiniae* (400 bps), *Meyerozymo guilliermondii* (300bps) and *Kluyveromyces marxianus* (200 bps). The bands for Mx2b, from top to bottom, represents *Diutina rugosa* (780 bps) and *Yarrowia lippolytica* (150 bps). The only bands not seen was that of *Deberomyces hansenii* (800 bps) and *Pichia norvegensis* (550 bps).

In figure 2.7c the PCR worked on all the available isolates from the third multiplex set. In the mixed sample the banding pattern, from top to bottom, represent *Trichosporon asahii* (500 bps),

Cryptococcus neoformans (400 bps), *Cryptococcus gattii* (200 bps) and *Rhodotorula mucliginosa* (100 bps).

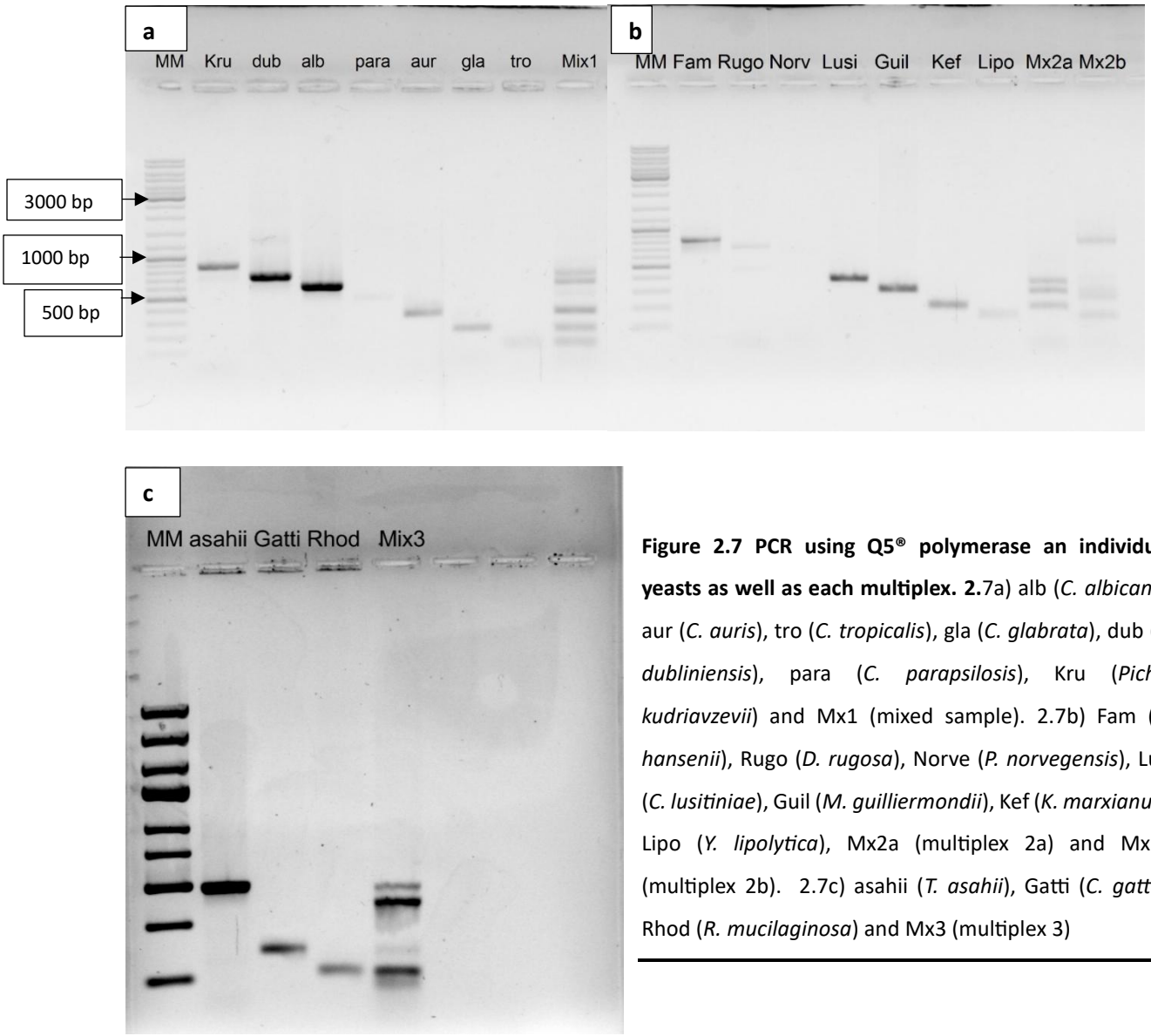


Figure 2.7 PCR using Q5® polymerase an individual yeasts as well as each multiplex. 2.7a) alb (*C. albicans*), aur (*C. auris*), tro (*C. tropicalis*), gla (*C. glabrata*), dub (*C. dubliniensis*), para (*C. parapsilosis*), Kru (*Pichia kudriavzevii*) and Mx1 (mixed sample). 2.7b) Fam (*D. hansenii*), Rugo (*D. rugosa*), Norve (*P. norvegensis*), Lusi (*C. lusitiniae*), Guil (*M. guilliermondii*), Kef (*K. marxianus*), Lipo (*Y. lipolytica*), Mx2a (multiplex 2a) and Mx2b (multiplex 2b). 2.7c) asahii (*T. asahii*), Gatti (*C. gattii*), Rhod (*R. mucilaginosa*) and Mx3 (multiplex 3)

There are several commercially available polymerases each with features that make them ideal for a wide range of applications. These polymerases are either isolated from microorganisms, for example Pfu which is isolated from *Pyrococcus furiosus*, (André et al., 1997) and *Taq*-polymerase isolated from *Thermus aquaticus* (Lawyer et al., 1989), or are produced through protein engineering, such as the Phusion® and Q5® polymerases. *Taq*-polymerase is the most commonly used (Yamagami et al., 2014)

but does not necessarily work for all applications. The fidelity associated with Pols are an essential factor for the accurate replication of a chosen nucleic acid template (Pezza et al., 2014). Q5®-polymerase has a fidelity of >280 times that of *Taq*-polymerase due to its ability to perform 3'-5' proofreading action (Pezza et al., 2014) which is absent in *Taq*-Pol. Proofreading would enable the polymerase to return to the nucleotide sequence to remove mismatched nucleotides to ensure the correct nucleotide order (Pezza et al., 2014; Kunkel, 2004). Q5®-polymerase is also fused with a processivity-enhancing Sso7d DNA binding domain (Menin and Nichols, 2013), which enables the polymerase to execute continuous DNA synthesis without dissociating from the DNA template before completion (the average number of bases incorporated before the polymerase dissociates from a template), this is done by maintaining the LF and thumb domain of the polymerase around the DNA template (Wu et al., 2017). Higher processivity further contributes to the higher fidelity associated with certain polymerases.

Different methods can be used to determine the error rate produced by different Pols such as the traditional blue-white screening (Cline et al., 1996; Barns, 1992) as well as high throughput Sanger sequencing (McInerney et al., 2014; Pezza et al., 2014) and variations thereof. An example is the use of PacBio single molecule real time (SMRT) sequencing, this was to determine and measure the errors that occurred during PCR using *Taq*-polymerase as well as other high-fidelity polymerases to compare the various polymerase's activity to one another (Potapov and Ong, 2017; Hestand et al., 2016). In this study the high-fidelity polymerase which is of interest is Q5®. *Taq*-polymerase showed to have an error rate of $1.8x10^{-4}$ errors/base while Q5®-polymerasel has a base substitution rate of only 5.3×10^{-7} sub/base, further highlighting the accuracy/fidelity at which Q5®-polymerase replicates DNA (Potapov and Ong, 2017). The error rate found from this study correspond to data from other articles which speculate a range of ~$1x10^{-4}$ to $2x10^{-5}$ errors/base in *Taq*-polymerase (Ricardo et al., 2020, McInerney et al., 2014).

HostStart DNA-Polymerases are also gaining popularity due to their advantages of room temperature setup and reduced non-specific amplification while enhancing replication efficiency and specificity (Cho et al., 1992). These DNA-polymerases are modified to be inactive at ambient temperatures through antibody interactions, chemical modifications or aptamer technology (NEB). Only when the reaction reaches a specific temperature will the DNA-polymerase dissociate from its inhibitors and begin the polymerisation of dNTPs into the replica sequence this minimises mispairing between the template strand and primers as well as the formation of primer dimers (Mi et al., 2009; Cho et al., 1992). The AccuStart II PCR ToughMix® used during optimisation contains an ultra-pure, highly

processive, hot start *Taq*-polymerase which in theory should have worked but did not yield the desired results.

In a study done by Arastehfar and colleagues, they successfully used *Taq*-polymerase to perform 3 multiplex PCR sets, using the same primers as in this study, yet we were not able to replicate their results using the same polymerase. The reason for this is still unclear as the same genomic DNA was used for the reactions using KAPA *Taq*-, AccuStart ToughMix and Q5®-polymerase and was performed under the same PCR conditions, yet the reaction using Q5®-polymerase performed the best. When comparing the results for the multiplex PCR done on the mixed samples from multiplex 1, the AccuStart reaction only yielded 3 (*C. albicans, C. glabrata* and *C. tropicalis*) out of the seven target bands while the Q5®-polymerase produced 6 (*C. dubliniensis, C. albicans, C. parapsilosis, C. auris, C. glabrata* and *C. tropicalis*. The only band not present is that of *Pichia kudriavzevii*) out of the 7 target bands. The high fidelity and processivity features of Q5®-polymerase may have been the determining factor to the results obtained. Thus, it was decided that this polymerase will be used for the continuation of multiplex PCR assays.

Multiplex PCR on wastewater sample

Since wastewater is a complex matrix, the multiplex PCR reactions also had to be optimized in this system. Multiplex 1, 2a, 2b and 3 PCR worked well on the test sample as seen with the formation of multiples bands corresponding to the ladders (Figure 2.8). There were however still non-specific binding products that formed at the bottom of the gel.

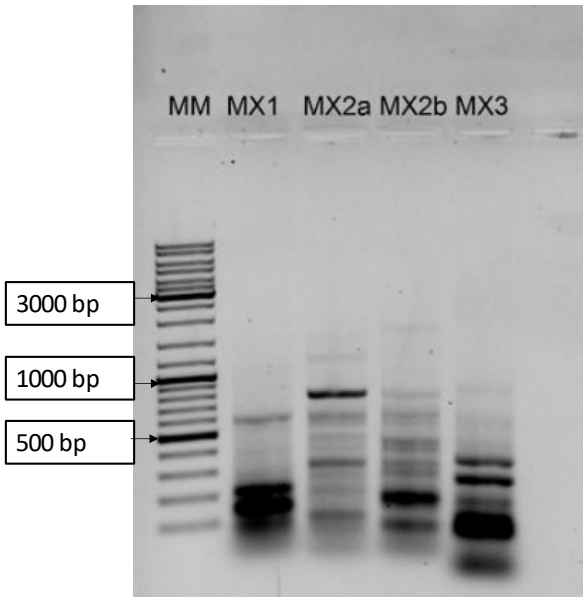


Figure 2.8 PCR using Q5® polymerase trial run on extracted DNA from a sewage sample. The multiplex from all 3 sets (Mx1, Mx2a, Mx2b ansd Mx3) was preformed

To determine if the yeast identified in a sample could in fact pose a health risk, only the DNA of the viable cells need to be amplified (Reyneke et al., 2017). Thus, it is essential to find ways to differentiate between the DNA of viable cells versus that of dead cells or extracellular DNA. This can be achieved by using a membrane-impermeable nucleic acid binding dye such as propidium monoazide (PMA) or EMA. These photoactivated dyes will covalently bind to the DNA of cells with damaged membranes as well as any extracellular DNA. When the dye is exposed to high-intensity light it forms a reactive nitrene which is capable of binding to DNA, thus preventing their amplification during PCR (Reyneke et al., 2017; Bellehumeur et al., 2015; Fakhri, 2014; Rawsthorne and Phister, 2009; Pisz et al., 2007). In addition, during genomic DNA extraction, the dye-conjugated DNA will be included in the cell debris pellet formed after centrifugation, following the initial cell lysis step, thus removing the non-viable cell's DNA from the total DNA extracted during the process (Nocker and Camper, 2006). The use of membrane impermeable dyes can prove useful in elucidating the presence of viable pathogenic yeast present in wastewater.

Looking at the gel electrophoresis results between the EMA treated and untreated wastewater samples, the EMA treated samples produced the clearest results. Figure 2.9a (treated samples) shows

clear, definite bands whereas figure 2.9b (untreated samples) shows smears, making it difficult to determine band sizes and reducing the accuracy of result interpretation.

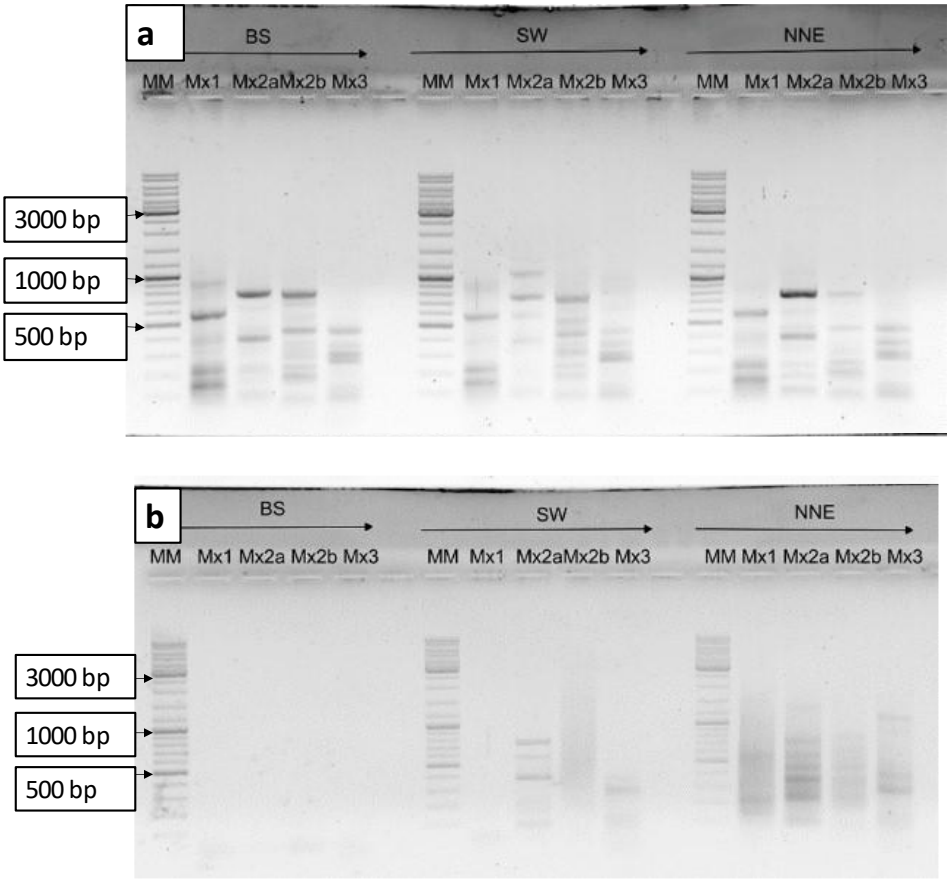


Figure 2.9 Comparison of a multiplex PCR performed on sewage samples 2.9a) treated with ethidium bromide monoazide and 2.9b) untreated samples. BS, SW and NNE are abbreviations used for the different wastewater treatment plants used in this study which will be explained in the next chapter

Some studies do however report that EMA inhibits the amplification of viable cell DNA (Cawthorn and Witthuhn, 2008; Pisz et al., 2007). It is speculated that EMA may still penetrate the membranes of viable cells but to a lesser extent than that of dead cells. Also, the intense light exposure may cause partial lysis to the membranes of cells causing them to be more susceptible to EMA (Nocker and Camper, 2006). A review article written by Fittipaldi et al., (2012) stated that the three main factors that may influence the penetration of PMA or EMA into viable cells are the concentration of the dye, incubation time and temperature. In this article, the authors concluded that high-concentration dyes should be incubated for a shorter time compared to low-concentration dyes. Incubation temperature may also influence the membrane permeability of cells thus it would be more efficient to incubate

samples at lower temperatures or on ice (Fittipaldi et al., 2012). Considering the methodology used in our study a 6 µM concentration of EMA was used for an incubation time of 20 minutes while the samples were on ice. These conditions proved optimal to reduce the occurrence of viable cell penetration (Reyneke et al. 2017).

Final optimised multiplex PCR reactions

The primers designed by Arastehfar and colleagues (2019) were mapped to the target sequences provided in the same article. From these results (supplementary data), we could see that within a few target sequences, multiple primers of the same multiplex set, could bind and ultimately amplify the same sequence during PCR. This might lead to additional problems when it comes to interpreting gel electrophoresis images as well as sequencing results. To reiterate on a previous statement, this tool was initially designed for clinical samples, where one would not expect to see several pathogenic yeast species in one sample/infection whereas wastewater samples contain a collection of fungal species along with a mixture of various inhibitors that may interfere with the efficiency of molecular analysis. Considering this information, we were able to explain some of the results that may cause confusion.

Looking at the gel electrophoresis images additional unexpected band varying in size can be observed. This was especially seen in multiplex 2a and 2b. Multiplex 2a was designed to only target 3 non-major *Candida* species, yet multiple bands can be observed at sizes much greater that the largest expected band size. After primer mapping, it was clear that due to the binding of multiple primers to the same complement sequences, primers for the specific multiplex set would be able to bind to target sequence for a different species to amplify that genetic material. The ratio in which different species occur in samples can also influence results. Higher occurrence of a specific yeast species to which multiple primers can bind, will outcompete binding to other species occurring at a lower concentration. Thus, primers designed for one specific species can amplify non-target sequences leading to additional bands, and a false positive if the target species for each multiplex set is not considered.

The following is an example of this occurrence: from the supplementary data it is seen that multiple primers can bind to *Debaromyces hanseni*'s sequence including primers targeting *Duitina rugosa* and *Meyerozyma guilliermondii* leading to the amplification of sequences of ~ 800 bp during the multiplex 2a PCR. So, within multiplex 2a it would seem that *Debaromyces hansenii* is also amplified but it might well be the primers of *Meyerozyma guilliermondii* binding to the compliment sequence found within *Debaromyces hanseni's* geneomic DNA. To conclude this section, when interpreting the multiplex PCR results, only the expected band sizes for each multiplex sets should be considered a positive result

even though bands of other sizes may occur, this could be due to non-specific binding of primers in such a complex sample. If a band occurs within multiplex 2a that corresponds to the expected size for one of the three target yeasts, it can be assumed as a positive detection (the same assumption should be made for the other multiplex sets). Future considerations should include designing primers more specific for each target isolate to prevent cross-binding of primers to the same targets.

Conclusions

This study investigated the efficiency of various methods for the isolation, genomic DNA extraction as well as the optimal PCR conditions needed to perform a multiplex PCR on wastewater samples. Considering the complexity of these samples, optimised methods for the analysis of wastewater are essential for the effective investigation of these samples. It was determined that yeast isolation using the supernatant of wastewater samples yielded the best results with little to no filamentous fungal growth observed on the selected medium. The combined use of wastewater supernatant and the supplementation of SDA with the broad-spectrum antibiotic, chloramphenicol, was sufficient in inhibiting the growth of bacteria along with filamentous fungi which may hinder our ability to isolate yeast colonies. To address the issue of the amplification of both viable and non-viable cells' DNA during PCR amplification, an initial EMA treatment was included.

Genomic DNA was extracted using various commercially available kits each with different features that make them ideal for extraction of DNA from various sample types. The OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit provided the best results which may be attributed to a silica bead-based binding column and an isopropanol precipitation step. The multiplex PCRs targeting various pathogenic yeast species, were also tested using three different commercially available DNA-polymerases as well as different salt concentrations and annealing temperatures. The Q5 DNA-polymerase worked best, possibly due to its high fidelity and processivity. It was also determined that the salt concentration used in the reaction did not need adjusting and that an annealing temperature of 50°C provided optimal universal conditions for the amplification of gene targets of numerous pathogenic yeast species in one reaction.

The optimised methods determined in this chapter will be used in chapter three for further studies in order to investigate the potential use of wastewater surveillance to predict yeast infections.

References

Aboutalebian S, Ahmadikia K, Fakhim H, Chabavizadeh J, Okhovat A, Nikaeen M and Mirhendi H (2021) Direct Detection and Identification of the Most Common Bacteria and Fungi Causing Otitis Externa by a Stepwise Multiplex PCR. Front Cell Infect Microbiol 11: 644060. doi: 10.3389/fcimb.2021.644060

Acharya KR, Dhand NK, Whittington RJ, Plain KM (2017) PCR Inhibition of a Quantitative PCR for Detection of Mycobacterium avium Subspecies Paratuberculosis DNA in Feces: Diagnostic Implications and Potential Solutions. Front Microbiol 8: 115. doi: 10.3389/fmicb.2017.00115

Ahmed W, Bivins A, Bertsch PM, Bibby K, Choi PM, Gyawali P, Hamilton KA, Haramoto E, Kitajima M, Simpson SL, Tandukar S (2020) Surveillance of SARS-CoV-2 RNA in wastewater: Methods optimization and quality control are crucial for generating reliable public health information. Curr Opin Environ Sci Health 17: 82-93. doi: https://doi.org/10.1016/j.coesh.2020.09.003

Ahmed W, Simpson SL, Bertsch PM, et al., (2022) Minimizing errors in RT-PCR detection and quantification of SARS-CoV-2 RNA for wastewater surveillance. Scie Total Environ 805: 149877. doi: https://doi.org/10.1016/j.scitotenv.2021.149877

Ali E, Razzak A, Hamid SBA (2014) Multiplex PCR in Species Authentication: Probability and Prospects—A Review. Food Anal. Methods 7: 1933–1949. doi: 10.1007/s12161-014-9844-4

André P, Kim A, Khrapko K, Thilly WG (1997) Fidelity and mutational spectrum of Pfu DNA polymerase on a human mitochondrial DNA sequence. Genome Res 7: 843-52. doi: 10.1101/gr.7.8.843

Arvanitis M, Anagnostou T, Fuchs BB, Caliendo AM, Mylonakisa E (2014) Molecular and Nonmolecular Diagnostic Methods for Invasive Fungal Infections. CMR 27: 490 –526. doi: https://doi.org/10.1128/cmr.00091-13

Arastehfar A, Fang W, Pan W, Lackner M, Liao W, Badiee P, Zomorodian K, Badali H, Hagen F, Lass-Flörl C, Boekhout T (2019) YEAST PANEL multiplex PCR for identification of clinically important yeast species: stepwise diagnostic strategy, useful for developing countries. Diagn Microbiol Infect Dis 93: 112-119. doi: https://doi.org/10.1016/j.diagmicrobio.2018.09.007

Arroyo MG, Ferreira AM, Frota OP, Brizzotti-Mazuchi NS, Peresi JTM, Rigotti MA, Macedo CE, de Sousa AFL, de Andrade D, de Almeida MTG (2020) Broad diversity of fungi in hospital water. Sci World J 2020: 9358542. doi: https://doi.org/10.1155/2020/9358542

Babič MN, Zalar P, Zenko B, D_zeroski, Gunde-Cimerman N (2016) Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances. Fungal Ecol 20: 30-39. doi: http://dx.doi.org/10.1016/j.funeco.2015.10.001

Babler K, Sharkey M, Arenas S, Amirali A, Baver C, Comerford S, Goodman K, Grills G, Holung M, Kobetz E, Laine J, Lamar W, Mason C, Pronty D, Reding B, Schürer S, Solle NS, Stevenson M, Vidović D, Solo-Gabriele H, Shukla B (2023) Detection of the clinically persistent, pathogenic yeast spp. Candida auris from hospital and municipal wastewater in Miami-Dade County, Florida. Sci Total Environ 898: 165459. doi: https://doi.org/10.1016/j.scitotenv.2023.165459

Barns WM (1992) The fidelity of *Taq* polymerase catalyzing PCR is improved by an N-terminal deletion. Gene 112: 29-35. doi: https://doi.org/10.1016/0378-1119(92)90299-5

Bellehumeur C, Boyle B, Charette SJ, Harel J, L'Homme Y, Masson L, Gagnon CA (2015) Propidium monoazide (PMA) and ethidium bromide monoazide (EMA) improve DNA array and high-throughput sequencing of porcine reproductive and respiratory syndrome virus identification. J virol methods *222*: 182-191. doi: http://dx.doi.org/10.1016/j.jviromet.2015.06.014

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases – estimate precision. J Fungi (Basel) 3: 57. doi: 10.3390/jof3040057

Birch DE (1996) Simplified hot start PCR. Nature 381: 445-446. doi: 10.1038/381445a0

Carraro L, Maifreni M, Bartolomeoli I, Martino ME, Novelli E, Frigo F, Marino M, Cardazzo B (2011) Comparison of culture-dependent and-independent methods for bacterial community monitoring during Montasio cheese manufacturing. Res Microbiol 162: 231-239. doi: https://doi.org/10.1016/j.resmic.2011.01.002

Cassinotti P, Siegl G (1998) A nested-PCR assay for the simultaneous amplification of HSV-1, HSV-2, and HCMV genomes in patients with presumed herpetic CNS infection. J Virol Methods 71**:** 105–114. doi: 10.1016/s0166-0934(97)00203-6

Caicedo-Bejarano LD, Osorio-Vanegas LS, Ramírez-Castrillón M, Castillo JE, Martínez-Garay CA, Chávez-Vivas M (2023) Water Quality, Heavy Metals, and Antifungal Susceptibility to Fluconazole of Yeasts fromWater Systems. Int J Environ Res Public Health 20: 3428. doi: https:// doi.org/10.3390/ijerph20043428

Cawthorn DM and Withuhn RC (2008) Selective PCR detection of viable Enterobacter sakazakii cells utilizing propidium monoazide or ethidium bromide monoazide. J Appl Microbiol 105: 1178-1185. doi:10.1111/j.1365-2672.2008.03851.x

Choi J, Marks J, Zhang J, Chen DH, Wang J, Vázquez-Laslop N, Mankin AS, Puglisi JD (2020) Dynamics of the context-specific translation arrest by chloramphenicol and linezolid. Nat Chem Biol 16: 310–317. doi: https://doi.org/10.1038/s41589-019-0423-2

Cline J, Braman JC, Hogrefe HH (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. Nucleic Acids Res 24: 3546-3551. doi: 10.1093/nar/24.18.3546

Da Silva RM, Da Silva Neto JR, Santos CS, Frickmann H, Poppert S, Santana Cruz K, Koshikene D, De Souza JVB (2015) Evaluation of fluorescence in situ hybridisation (FISH) for the detection of fungi directly from blood cultures and cerebrospinal fluid from patients with suspected invasive mycoses. Ann Clin Microbiol Antimicrob 14: 6. doi: https://doi.org/10.1186/s12941-015-0065-5

Denning DW (2017) Calling upon all public health mycologists. Eur J Clin Microbiol Infect Dis 36: 923–924. doi: https://doi.org/10.1007/s10096-017-2909-8

Dinos GP, Athanassopoulos CM, Missiri DA, Giannopoulou PC, Vlachogiannis IA, Papadopoulos GE, Papaioannou D, Kalpaxis DL (2016) Chloramphenicol Derivatives as Antibacterial and Anticancer Agents: Historic Problems and Current Solutions. Antibiotics 5: 20. doi: 10.3390/antibiotics5020020

Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE (2000) Multiplex PCR: optimization and application in diagnostic virology. CMR 13: 559-570. doi: https://doi.org/10.1128/cmr.13.4.559

Endres HN, Johnson JAC, Ross CA, Welp JK, Etzel MR (2003). Evaluation of an ion-exchange membrane for the purification of plasmid DNA. Biotechnol Appl Biochem 37: 259-266. doi:10.1042/ba20030025

Fakhri A (2014) Assessment of Ethidium bromide and Ethidium monoazide bromide removal from aqueous matrices by adsorption on cupric oxide nanoparticles. Etoxicol Environ Saf 104: 386-392. doi: https://doi.org/10.1016/j.ecoenv.2013.12.017

Farkas K, McDonald JE, Malham SK, Jones DL (2018) Two-Step Concentration of Complex Water Samples for the Detection of Viruses. Methods Protoc 10: 35. doi: 10.3390/mps1030035

Fittipaldi M, Nocker A, Codony F (2012) Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J Microbiol Meth 91: 276–289. doi: https:// doi.org/10.1016/j.mimet.2012.08.007

Fishman JA (2020) *Pneumocystis jiroveci*. Semin Respir Crit 41: 141-157. doi: 10.1055/s-0039-3399559

Hachem RY, Kontoyiannis DP, Chemaly RF, Jiang Y, Reitzel R, Raad I (2009) Utility of galactomannan enzyme immunoassay and (1,3) b-D-glucan in diagnosis of invasive fungal infections: low sensitivity for *Aspergillus fumigatus* infection in hematologic malignancy patients. J Clin Microbiol 47: 129–133. doi: https://doi.org/10.1128/jcm.00506-08

Hestand MS, Van Houdt J, Cristofoli F, Vermeesch JR (2016) Polymerase specific error rates and profiles identified by single molecule sequencing. Mutant Res 784-785: 39-45. doi: http://dx.doi.org/10.1016/j.mrfmmm.2016.01.003 0027-5107

Harrigan WF, McCance ME (1966) Laboratory Methods in Microbiology. Academic Press, London

Huang Q, Yu Y, Tang C, Peng X (2010) Determination of commonly used azole antifungals in various waters and sewage sludge using ultra-high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A 1217: 3481-3488. doi: https://doi.org/10.1016/j.chroma.2010.03.022

Jones OAH, Voulvoulis N, Lester JN (2005) Human Pharmaceuticals in Wastewater Treatment Processes. Crit Rev Environ Sci Technol 35: 401-427. doi: 10.1080/10643380590956966

Juel MAI, Stark N, Nicolosi B, Lontai J, Lambirth K, Schlueter J, Gibas C, Munir M (2021) Performance evaluation of virus concentration methods for implementing SARS-CoV-2 wastewater based epidemiology emphasizing quick data turnaround. Sci Total Environ 20;801: 149656. doi: 10.1016/j.scitotenv.2021.149656

Kainz K, Bauer MA, Madeo F, Carmona-Gutierrez D (2020) Fungal infections in humans: the silent crisis. Microbial cell 7: 143-145. doi: 10.15698/mic2020.06.718

Kedzierska A, Kochan P, Pietrzyk A, Kedzierska J (2007) Current status of fungal cell wall components in the immunodiagnostics of invasive fungal infections in humans: galactomannan, mannan and (1A3)-b-D-glucan antigens. Eur J Clin Microbiol Infect Dis 26: 755–766. doi: 10.1007/s10096-007-0373-6

Kosmidis C, Denning DW (2015) The clinical spectrum of pulmonary aspergillosis. Thorax 70**:** 270-277. doi: 10.1136/thoraxjnl-2014-206291

Kumblathan T, Piroddi N, Hrudey SE, Li X-F (2022) Wastewater Based Surveillance of SARS-CoV-2: Challenges and Perspective from a Canadian Inter-laboratory Study. J Environ Sci 116: 229-232. doi: https://doi.org/10.1016/j.jes.2022.01.039

Kunkel TA (2004) DNA Replication Fidelity. J Biol Chem 279: 16895–16898. doi: 10.1074/jbc.R400006200

Lawyer FC, Stoffel S, Saiki RK, Myambo K, Drummond R, Gelfand DH (1989) Isolation, characterization, and expression in Escherichia coli of the DNA polymerase gene from Thermus aquaticus. J Biol Chem 264: 6427-6437. doi: https://doi.org/10.1016/S0021-9258(18)83367-1

Lindberg RH, Fick J, Tysklind M (2010) Screening of antimycotics in Swedish sewage treatment plants – Waters and sludge. Water Res 44: 649-657. doi: https://doi.org/10.1016/j.watres.2009.10.034

Mahony J, Chong S, Merante F, Yaghoubian S, Sinha T, Lisle C, Janeczko R (2007) Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay. J Clin Micriobiol 45: 2965-2970. doi: https://doi.org/10.1128/jcm.02436-06

McLain JE, Cytryn E, Durso LM, Young S (2016), Culture-based Methods for Detection of Antibiotic Resistance in Agroecosystems: Advantages, Challenges, and Gaps in Knowledge. J Environ Qual 45: 432-440. doi: https://doi.org/10.2134/jeq2015.06.0317

McInerney P, Adams P, Hadi MZ (2014) Error Rate Comparison during Polymerase Chain Reaction by DNA Polymerase. Mol Biol Int 2014: 287430. doi: 10.1155/2014/287430

Medeiros AO, Missagia BS, Brandão LR, Callisto M, Barbosa FAR, Rosa CA (2012) Water quality and diversity of yeast from tropical lakes and rivers from the Rio Doce basin in Southeastern Brazil. Braz J Microbiol 43: 1582-1594. doi: https://doi.org/10.1590/S1517-83822012000400043

Menin JF and Nichols NM (2013) Multiplex PCR using Q5® High-Fidelity DNA Polymerase. N Engl Biolabs Appl. Note.

Mi L, Wen Y, Pan D, Wang Y, Fan C, Hu J (2009) Modulation of DNA Polymerases with Gold Nanoparticles and their Applications in Hot-Start PCR. Small 5: 2597-2600. doi: https://doi.org/10.1002/smll.200901147

Moragues MD, Ortiz N, Iruretagoyena JR, Garcia-Ruiz JC, Amutio E, Rojas A, Mendoza J, Quindos G, Ponton-San Emeterio J (2004) Evaluation of a new commercial test (*Candida albicans* IFA IgG) for the serodiagnosis of invasive candidiasis. Enferm Infecc Microbiol Clin 22: 83–88. doi: http://dx.doi.org/10.1016/S0213-005X(04)73039-6

New England Bioliabs®Inc. Hot Start PCR. https://international.neb.com/applications/dna-amplification-pcr-and-qpcr/specialty-pcr/hot-start-pcr#:~:text=Hot%20Start%20PCR%20is%20a,are%20unreactive%20at%20ambient%20temperatures. Accessed 18 July 2023

Nicula NO, Lungulescu EM, Rîmbu GA, Marinescu V, Corbu VM, Csutak O (2023) Bioremediation of Wastewater Using Yeast Strains: An Assessment of Contaminant Removal Efficiency. Int J Environ Res Public Health 20: 4795. doi: https://doi.org/10.3390/ijerph20064795

Nocker A, Camper AK (2006) Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl Environ Microbiol 72: 1997–2004. doi: https://doi.org/10.1128/AEM.72.3.1997-2004.2006

Nugaeva N, Gfeller KY, Backmann N, Lang HP, Düggelin M, Hegner M (2005) Micromechanical cantilever array sensors for selective fungal immobilization and fast growth detection. Biosens Bioelectron 21: 849-856. doi: https://doi.org/10.1016/j.bios.2005.02.004

Parslow BY and Thornton CR (2022) Continuing shifts in epidemiology and antifungal susceptibility highlight the need for improved disease management of invasive Candidiasis. Microorganisms 10: 1208. doi: 10.3390/microorganisms10061208

Patel R (2015) MALDI-TOF MS for the Diagnosis of Infectious Diseases. Clin Chem 61: 100–111. doi: https://doi.org/10.1373/clinchem.2014.221770

Pegorie M, Denning DW, Welfare W (2017) Estimating the burden of invasive and serious fungal disease in the United Kingdom. J Infect 74: 60-71. doi: https://doi.org/10.1016/j.jinf.2016.10.005

Persson K, Hamby K, Ugozzoli LA (2005) Four-color multiplex reverse transcription polymerase chain reaction—Overcoming its limitations. Anal Biochem 344: 33-42. doi: 10.1016/j.ab.2005.06.026

Pereira VJ, Basıˊlio MC, Fernandes D, Domingues M, Paiva JM, Benoliel MJ, Crespo MT, San Roma˜o MV (2009) Occurrence of filamentous fungi and yeasts in three different drinking water sources. Water Res 43: 3813–3819. doi: 10.1016/j.watres.2009.05.024

Pezza JA, Kucera R, Sun L (2014) Polymerase fidelity: what is it, and what does it mean for your PCR. *New England Biolabs*.

Pisz JM, Lawrence JR, Schafer AN, Siciliano SD (2007) Differentiation of genes extracted from non-viable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. J Microbiol Methods 71: 312-318. doi: 10.1016/j.mimet.2007.09.015

Pongs O (1979) Chloramphenicol. In: Hahn, F.E. (eds) Mechanism of Action of Antibacterial Agents. Antibiotics, vol 5 / 1. Springer, Berlin, Heidelberg. doi: https://doi.org/10.1007/978-3-642-46403-4_3

Potapov V, Ong JL (2017) Examining Sources of Error in PCR by Single-Molecule Sequencing. PLoS ONE 12: e0169774. doi: 10.1371/journal.pone.0169774

Qdais HA, Moussa H (2004) Removal of heavy metals from wastewater by membrane processes: a comparative study. Desalination 154: 105-110. doi: https://doi.org/10.1016/S0011-9164(04)00169-9

Racil Z, Kocmanova I, Lengerova M, Weinbergerova B, Buresova L, Toskova M, Winterova J, Timilsina S, Rodriguez I, Mayer J (2010) Difficulties in using 1,3-b-D-glucan as the screening test for the early diagnosis of invasive fungal infections in patients with haematological malignancies – high frequency of false-positive results and their analysis. J Med Microbiol 59: 1016–1022. doi: 10.1099/jmm.0.019299-0

Rajkumari N, Mathur P, Xess I, Misra M (2014) Distribution of different yeasts isolates among trauma patients and comparison of accuracy in identification of yeasts by automated method versus conventional methods for better use in low resource countries. Indian J Med Microbiol 32: 391. doi: https://doi.org/10.4103/0255-0857.142243

Rajasingham R, Govender NP, Jordan A, Loyse A, Shroufi A, Denning DW, Meya DB, Chiller TM, Boulware DR (2022) The global burden of HIV-associated cryptococcal infection in adults in 2020: a modelling analysis. Lancet Infect Dis 22: 1748-1755 doi: https:/doi.org/10.1016/S1473-3099(22)00499-6

Rawsthorne H and Phister TG (2009) Detection of viable Zygosaccharomyces bailii in fruit juices using ethidium monoazide bromide and real-time PCR. Int J Food Microbiol 131: 246–250. doi: 10.1016/j.ijfoodmicro.2009.01.031

Reyneke B, Ndlovu T, Khan S, Khan W (2017) Comparison of EMA-, PMA- and DNase qPCR for the determination of microbial cell viability. Appl Microbiol Biotechnol 101: 7371–7383. doi: 10.1007/s00253-017-8471-6

Ricardo PC, Françoso E, Arias MC (2020) Fidelity of DNA polymerases in the detection of intraindividual variation of mitochondrial DNA. Mitochondrial DNA Part B 5: 108–112. doi: https://doi.org/10.1080/23802359.2019.1697188

Schrader C, Schielke A, Ellerbroek L, Johne R (2012) PCR inhibitors - occurrence, properties and removal. J Appl Microbiol 113: 1014–1026. doi: doi:10.1111/j.1365-2672.2012.05384.x

Sharma M, Chakrabarti A (2023) Candidiasis and Other Emerging Yeasts. Curr Fungal Infect Rep 17: 15–24. doi: https://doi.org/10.1007/s12281-023-00455-3

Shi W, Zhuang WE, Hur J, Yang L (2021) Monitoring dissolved organic matter in wastewater and drinking water treatments using spectroscopic analysis and ultra-high resolution mass spectrometry. Water Res 188: 116406. doi: https://doi.org/10.1016/j.watres.2020.116406

Sidstedt M, Rådström P, Hedman J (2020) PCR inhibition in qPCR, dPCR and MPS-mechanisms and solutions. Anal Bioanal Chem 412: 2009-2023. doi: 10.1007/s00216-020-02490-2

Stevens DA (2002) Diagnosis of fungal infections: current status. J Antimicrob Chemother 49: 11-19. doi: 10.1093/jac/49.suppl_1.11

Taniuchi M, Verweij JJ, Noor Z, Sobuz SU, van Lieshout L, Petri Jr. WA, Haque R, Houpt ER (2011) High Throughput Multiplex PCR and Probe-based Detection with Luminex Beads for Seven Intestinal Parasites. Am J Trop Med Hyg 84: 332–337. doi:10.4269/ajtmh.2011.10-0461

Van Wyk DAB, Bezuidenhout CC, Rhode OHJ (2012) Diversity and characteristics of yeasts from water sources

in the North West Province, South Africa. Water Sci Technol: Water Supply 12: 422-430. doi: https://doi.org/10.2166/ws.2012.009

WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO.

Widjojoatmodjo MN, Borst A, Schukkink RA, Box AT, Tacken NM, Van Gemen B, Verhoef J, Top B, Fluit AC (1999) Nucleic acid sequence-based amplification (NASBA) detection of medically important *Candida* species. J Microbiol Methods 38: 81-90. doi: https://doi.org/10.1016/S0167-7012(99)00079-2

Wu J, de Paz A, Zamft BM, Marblestone AH, Boyden ES, Kording KP, Tyo KEJ (2017) DNA binding strength increases the processivity and activity of a Y-Family DNA polymerase. Sci Rep 7: 4756. doi: https://doi.org/10.1038/s41598-017-02578-3

Yamagami T, Ishino S, Kawarabayasi Y, Ishino Y (2014) Mutant Taq DNA polymerases with improved elongation ability as a useful reagent for genetic engineering. Front Microbiol 5: 461. doi: https://doi.org/10.3389/fmicb.2014.00461

Yang Y, Hebron HR, Hang J (2008) High performance DNA purification using a novel ion exchange matrix. J Biomol Tech 19: 205-10.

Chapter 3:

Application of a wastewater surveillance system to the detection of pathogenic yeast

(Parts of this chapter have been submitted for publication)

Baker T, Bester PA, Sebolai OM, Albertyn J, Pohl CH (2025) Culture-Dependentand -Independent Wastewater Surveillance for Multiple Pathogenic Yeasts. JFungi 11: 86. doi: https://doi.org/10.3390/jof11020086

Abstract

Wastewater surveillance/Wastewater-based epidemiology is well-developed for bacteria and viruses, but less so for the surveillance of pathogenic yeast. This system could prove useful in determining what fungal infections are prominent in various communities. In this study, we investigated the presence of pathogenic yeasts in the wastewater influent of six different sampling points in Bloemfontein, South Africa. From these samples, yeasts were isolated and identified and it was observed that *Candida* species were the most prominent. A multiplex PCR system (optimised in Chapter 2) was used and found to be able to identify various pathogenic yeast species, which largely corresponded with the culture-dependent results. Growth studies were also performed using wastewater as a medium to prove the concept that wastewater can act as a hub of yeast growth and proliferation. Finally, the culture-dependent and independent data was compared to cases of fungal infections reported by public hospitals in the area. Unfortunately, inherent weaknesses in the hospital data made it impossible to draw any correlations.

Keywords: Multiplex PCR, Pathogenic yeast, Sanger sequencing

Introduction

The development of accurate and efficient systems for the surveillance of infectious diseases is a critical factor that influences the response and preparation of the healthcare sector towards disease outbreaks (McCall et al., 2020). The ability to detect potential outbreaks, before most of the community or population is infected, holds various advantages, such as timeliness of disease detection and reporting as well as efficient treatment, thus reducing mortality rates associated with the detected disease. One method that shows continuous promise for predicting outbreaks is wastewater surveillance or wastewater-based epidemiology (WBE). This system can act as an early warning system, while also allowing for the unbiased assessment of the disease burden and transmission in a catchment area (Brumfield et al., 2022; McCall et al., 2020). Recently, WBE has been used to detect outbreaks of polio (Wise 2022; Anis et al. 2013; Paul et al. 1939) and COVID-19 (Brumfield et al. 2022; Wu et al. 2021) and has a history of being used to monitor the occurrence of potentially harmful or enteric viruses such as hepatitis, noro- and entero-viruses as well as cholera outbreaks (McCall et al., 2020; Tiwari and Dhole 2018). Figure 3.1 depicts the basic workflow followed for WBE studies.

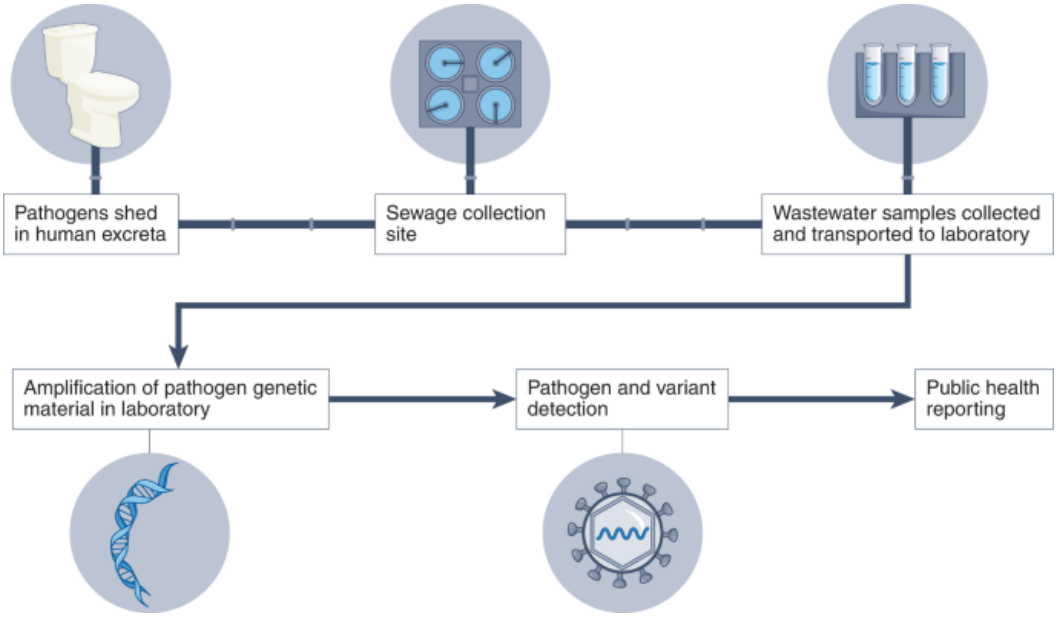


Figure 3.1 Basic workflow of wastewater-based epidemiology (WBE). Pathogens are shed into wastewater systems through human fecal matter as well as household surfaces. Wastewater samples are collected and sent to designated laboratories where genetic material is extracted and pathogens are identified using PCR and qPCR. The results obtained are then communicated to the relevant authorities and the public (Diamond et al., 2022)

Although WBE has become a common practice in the detection of viral and bacterial agents of disease, few attempts have been made to apply this surveillance system to the detection of pathogenic fungi (Zulli et al., 2024; Babler et al., 2023; Barber et al., 2023; Rossi et al., 2023). This lack of attention directed towards the surveillance of fungal pathogens circulating within the general community is concerning (Egger et al., 2022). Annually invasive fungal infections (Ifs) affect over 1 billion people worldwide, with over 2.5 million deaths (Kainz et al. 2020; Bongomin et al., 2017) causing more deaths than either tuberculosis or malaria. Due to the increasing incidences of fungal infections, partly driven by the increase in more aggressive therapeutic approaches, increase in cancer cases as well as the Covid-19 pandemic (Hoenigl et al., 2022; Moser et al., 2021; Bongomin et al., 2017), the World Health Organization (2022) published a list of priority fungal pathogens where yeast genera such as *Candida*, and *Cryptococcus* are found at all levels of this list.

Most reports on fungal infection incidences and strain occurrence are made through hospitals or clinics, thus only including individuals who seek medical care or those who can afford to travel to hospitals and receive treatment. This creates a biased overview of fungal infection incidences and serious attention, and efforts must be made to rectify this. A potential solution may be applying WBE to detect fungal pathogens, especially life-threatening pathogenic yeasts.

In this study, culture-dependent and -independent techniques were used to investigate the application WBE to pathogenic yeasts. A multiplex PCR (optimised in Chapter 2) containing species-specific primers to detect major *Candida* pathogens, minor *Candida* pathogens and non-*Candida* pathogens in wastewater from Bloemfontein, Mangaung Metro, South Africa, was used. In addition, yeasts were isolated and identified. The results were then compared to fungal infection incidences reported by public hospitals in the Mangaung Metro. Additionally, growth studies were performed as a proof of concept that wastewater can support the growth of pathogenic yeast.

Materials and methods

Sample collection

Sampling was done once a month from May 2023 to April 2024. Samples were collected from six different sites in Bloemfontein, Mangaung Metro, South Africa, as indicated in Table 3.1 and Figure 3.2, according to the protocol outlined by the South African Medical Research Council (SAMRC) (SAMRC, 2021). One liter of wastewater influent was collected after the first grit screen, stored in closed,

labelled containers during transport and kept in a 4°C refrigerator until use (within 2 hours after the last sample was collected).

Table 3.1 Sample locations and coordinates

Sample location (abbreviation)	Coordinates
Bainsvlei treatment works (BV)	29°06'04.3"S 26°06'54.2"E
Woodland Hills treatment works (WL)	29°02'42.0"S 26°12'03.6"E
Bloemwater treatment works (BW)	29°07'29.7"S 26°14'46.9"E
Sterkwater treatment works (SW)	29°11'22.8"S 26°18'28.8"E
North East treatment works (NNE)	29°05'28.3"S 26°19'21.7"E
University of the Free State (UFS)	29°06'58.4"S 26°11'26.0"E

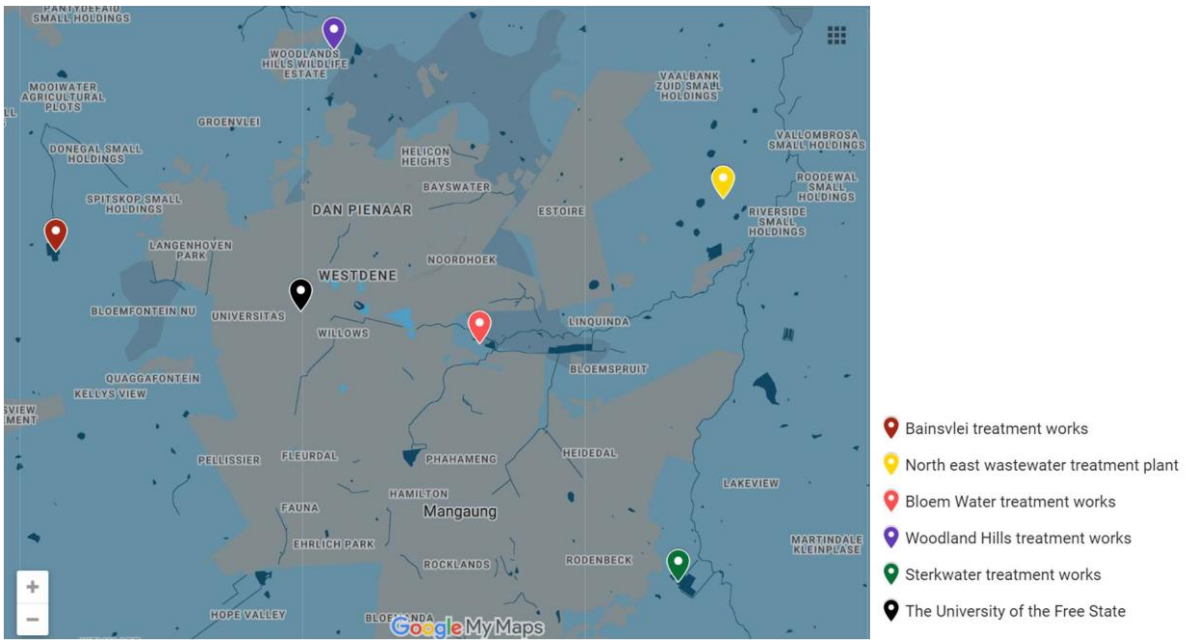


Figure 3.2 Map of the treatment works sampled in Bloemfontein, Mangaung Metro, South Africa. The lighter grey colour indicates the urban Bloemfontein area, whereas the blue indicates more rural, undeveloped areas outside of Bloemfontein

Sample location details:

1. The BV treatment plant (western parts of Bloemfontein) collects wastewater from middle-class households, small businesses and surrounding agricultural plots.
2. The WL Treatment plant (northern part of Bloemfontein close to the N1 national route) collects wastewater from a higher-income community, the military base, agricultural plots, and large fuel

stations on the N1. It is important to keep in mind that this sample pool includes samples from persons passing through Bloemfontein on the N1.

3. The BW treatment plant (central Bloemfontein) collects wastewater from most of the city, so this includes industrial, commercial and residential sources. This plant also has a collection damn for vacuum tankers to dispose of wastewater collected from septic tanks and other sources that do not deposit wastewater directly into established sewer systems.
4. The SW treatment plant (southern Bloemfontein close to an informal settlement) collects wastewater from agricultural plots, two high-security prisons and low-income to indigent communities.
5. The NE treatment plant (north-eastern Bloemfontein close to the Bram Fischer International Airport) collects wastewater from the airport, agricultural plots and small businesses in the area.
6. The UFS sampling point was a manhole on the university's Bloemfontein campus. Wastewater passing through this point is distributed to the BW treatment plant.

Samples could not be collected from UFS from January 2024 to April 2024 due to construction blocking the manhole used to access raw wastewater. SW treatment plant was not operational during June 2023 and October 2023 for various reasons, and no samples were collected from BV treatment plant for November 2023 due to load shedding.

Yeast isolation

Yeast isolation was performed every second month from May 2023 to April 2024. A 50 ml aliquot of wastewater was centrifuged at 7830 rpm (Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor) and the supernatant was serially diluted to 100 times. These dilutions were filtered using a 0.45 µm cellulose nitrate filter (Satorius; Germany) and the filter disks placed on Sabouraud dextrose agar, supplemented with chloramphenicol (10 g/L peptone powder, 40 g/L dextrose, 500 mg/L chloramphenicol, 15g/L bacteriological agar - Sigma Aldrich; Missouri USA) and incubated at 37°C for 48 hours. Six yeast colonies were selected using Harrison's Disc method (Harrigan & McCance, 1966) and further purified on yeast malt agar(YM) (3g/L yeast extract, 3g/L malt extract, 5g/L peptone powder, 10g/L glucose and 16 g/L bacteriological agar) incubated at 37°C. Colonies were confirmed to be yeast cells using microscopy and the isolates were deposited into the SANBI BBSA Yeast culture collection at the Department of Microbiology and Biochemistry, University of the Free State.

Molecular identification using ITS primers

For all pure cultures, colony PCR was performed. The end of a pipette tip was pressed into the fresh cultures (grown overnight) and resuspended into 10.5 µl of molecular-grade water. This mixture was boiled at 94°C for 10 minutes in a thermocycler (Applied biosystems 2720 Thermocycler). A 25 µl PCR reaction was made up for each isolate: 10.5 µl boiled culture solution, 1 µl ITS 4 primer (Integrated DNA Technologies; Iowa USA (IDT) -5'-TCCTCCGCTTATT3ATATGC-3'), 1µl ITS 5 primer (IDT 5'-GGAAGTAAAAGTCGTAACAAGG-3') and 12.5 µl Quantabio AccuStart II PCR ToughMix® [2X] (Massachusetts; USA). The PCR conditions were as follows: Initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 sec, Final extension at 72°C for 5 min and hold at 4°C. An 0.8% gel was run at 90 Volts for 30 min to confirm the amplification of the ITS sequence.

Clean-up and sequencing preparations were done twice for all samples, once with the ITS 4 primer as the sequencing primer and again using the ITS 5 primer as the sequencing primer. A volume of 5 µl of the ITS amplicon for each isolate was mixed with 1 µl [1u] FastAP™ Thermosensitive Alkaline Phosphatase (New England Biolabs, Massachusetts USA) and 0.5 µl [10u] Exonuclease I (Exo I) (New England Biolabs, Massachusetts USA) and incubated at 37°C for 15 min and a further 15 min at 85°C.

Clean-up products were prepared for sequencing using the Applied Biosystems BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems; Massachusetts USA) in which the protocol for a 1/16 size reaction (total of 10 µl reaction volume) was followed: 0.5 µl premix, 1 µl sequencing primer [3,2 pmol.μl^{-1}], 2 µl dilution buffer, 3 µl template and 3.5 µl molecular grade water. The provided control reaction (1/16 reaction size) was also made. The PCR conditions were as follows: Initial denaturation at 96°C for 1 min, denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, extension at 60°C for 4 min and a hold phase at 4°C, the reaction was set for 25 cycles.

For post-reaction clean-up, the 10 µl reaction volume was adjusted to 20 µl using molecular grade water. This was then transferred to 1.5 ml Eppendorf tubes containing 5 µl [125mM] EDTA and 60 µl absolute ethanol. The tubes were vortexed and left to precipitate at room temperature for 15 min, these tubes were then centrifuged at 20 000 *g* for 15 min at 4°C. The supernatant was completely aspirated without disturbing the pellet and 200 µl of 70% ethanol was added, the tubes were centrifuged at 20 000 *g* for 5 min at 4°C. The supernatant was then completely aspirated and the pellet was dried in a Speed-Vac (Eppendorf Concentrator Plus) for 5 min. Samples were stored at 4°C, protected from light.

Amplicons were sequenced using Applied biosystems™ 3500 genetic analyser. Geneious Prime version 2023.2.1 was used to construct a consensus sequence. This consensus sequence was identified using NCBI's BLAST function.

Growth studies

One representative yeast from each of the identified species was cultured overnight on yeast malt agar YM at 37°C for 24 hours. Isolates were standardised with sterile water to an optical density of 1 at 600nm ($1x10^{-6}$ cells/ml). Wastewater from the Bloemwater treatment plant was filtered using glass fibre (Schleicher and Schuell Bioscience, New Hampshire), 0.45µm nitrocellulose (Satorius; Germany) and 0.22 µm filter discs. Wastewater (90 µl)was dispensed into a 96-well plate followed by 10 µl of standardised sample (final concentration of $1x10^{-5}$ cells/ml). This was done in biological and technical triplicates. The Victor Nivo Multimode Microplate Reader (Revvity, Poland) was used to measure optical density (OD_{600nm}) every hour for 48 hours at 37°C, wavelength of 600 nm with orbital shaking before every measurement.

DNA extraction

A 5mg/ml stock of ethidium monoazide bromide (EMA) (Invetrogen-Thermo Fisher Scientific, Massachusetts USA), dissolved in a 20% dimethyl sulfoxide (Sigma ® Life Science, Missouri USA), was added to the 50 ml wastewater sample to a final concentration of 6 µM. Samples were vortexed and incubated horizontally on ice for 10 minutes in the absence of light. Incubation was followed by a 15-minute exposure to a halogen light source. Samples were washed with 1 ml 0.85% NaCl solution and centrifuged at 7830 rpm (Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor) after which the supernatant was discarded, and the remaining pellet was resuspended in 30 ml PBS (Reyneke et al., 2017).

DNA from living cells was extracted using the Omega Bio-Tek E.N.Z.A.® Water DNA kit (Georgia; USA). Wastewater (50 ml) was filtered through a 0.45 µm cellulose nitrate filter and DNA extraction performed according to the manufacturer's instructions with elution using 60 µl of elution buffer. Gel electrophoresis was used to confirm genomic DNA extraction (0.8% agarose gel at 90V, 400mA for 25 minutes).

Multiplex PCR

Four reactions using Q5® High-Fidelity DNA Polymerase (New England Biolabs; Massachusetts USA) were set up. Multiplex **1** contained the primers for *Candida albicans, C. auris*, *C. dubliniensis*, *C.*

tropicalis, *C. glabrata*, *C. parapsilosis* and *Pichia kudriavzevii.* Multiplex **2a** contained primers for *Myerozyma guiliermondii*, *Kluyveromyces marxianus* and *Clavispora lusitaniae.* Multiplex **2b** contained primers for *Debaromyces hansenii, Yarrowia lipolytica, Pichia norvegensis* and *Diutina rugosa*. Multiplex **3** contained primers for *Cryptococcus neoformans*, *Cryptococcus gattii*, *Rhodotorula mucliginosa* and *Trichosporon asahii.* Each reaction contained 5 µl eDNA template, 1 µl forward and reverse primer respectively, 1 µl of 10mM dNTPs, 0.5 µl Q5® High-Fidelity DNA Polymerase, 10 µl of a (5x) reaction buffer and molecular grade water up to 50µl final volume. PCR conditions were as follows: initial denaturation at 95°C for 5 minutes (1 cycle), denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 40 seconds (each for 35 cycles), and lastly final extension at 72°C for 8 minutes. PCR amplicons were visualised using gel electrophoresis, 1% agarose gel at 90V, 400mA for 30 minutes and identified based on amplicon size as specified in the article by Arastehfar and colleagues (2019). Primer sequences can be found under chapter 2 of the supplementary material.

Prevalence for each yeast species was calculated using the equation below:

$$\text{Prevalence (\%)} = \left(\frac{n}{total\ number\ of samples}\right) \times 100$$

*n = the number of samples containing a specific yeast species

Acquisition of weather data

Average maximum and minimum temperature as well as the total rainfall during the week preceding sampling were obtained from https://www.visualcrossing.com/weather-history/Bloemfontein/metric.

Acquisition of hospital data on fungal infections

Data on fungal infection incidences was obtained from the National Health Laboratory Service (NHLS) for the following hospitals in the Mangaung Metro: Universitas hospital, Universitas hospital annex, Pelonomi hospital, NHLS QA Free State Academic (CE), National district hospital and 3 Military hospital. This data included the name of the lab where samples were tested, the unique patient and episode identification number, the facility from which the samples were collected, the date tested, method used for testing and the name of the organisms detected. For this study, only cases caused by pathogenic yeasts were considered. Full details can be seen in table S2 in The supplementary material.

Results and discussion

Culture-dependant surveillance indicates abundant pathogenic *Candida* species

One hundred and eighty six isolates were obtained of which 20 were found to be bacteria. Following the identification of the 166 yeast isolates (Table S1 – data for percentage identity), it was found that *Candida* species were most commonly isolated and made up 43.98% of the total isolates (Table 3.2). This corresponds to previous studies investigating fungal occurrence in various freshwater sources as well as tap water (Monapathi et al., 2021; Pour et al., 2018; Babič et al., 2016; Medeiros et al., 2012; Pereira et al., 2009; Yamaguchi et al., 2007). In these studies, the most abundant *Candida* species were *C. tropicalis* (Monapathi et al., 2021; Ayanbimpe et al., 2012) and *C. parapsilosis* (Babič et al., 2016; Yamaguchi et al., 2007). Studies investigating fungal presence in wastewater tend to focus on species occurring in activated sludge and found *C. albicans* and *C. krusei* (*Pichia kudriavzevii*) (Cooke and Pipes 1969; Cooke 1970; Kacprzak et al., 2005; Yang et al., 2013; Cooke and Pipes 1969).

Table 3.2: Names and quantity of yeasts isolated from May 2023 to March 2024

Species	May	July	September	November	January	March	Total no. of isolates
Candida albicans	4	3	6	0	2	3	18
Candida glabrata	11	6	8	3	3	10	41
Candida palmioleophila	1	1	0	0	0	0	2
Candida parapsilosis	0	0	0	0	1	0	1
Candida stellimalicola	0	1	0	0	0	0	1
Candida tropicalis	0	3	1	0	4	0	8
Candida sp.	0	0	2	0	0	0	2
Clavispora lusitaniae	5	0	0	0	1	0	6
Dipodascus capitatus	0	0	0	1	0	0	1
Exophiala dermatitidis	0	0	0	1	0	0	1
Hanseniaspora pseudoguilliermondii	0	0	1	0	0	0	1
Magnusiomyces capitatus	0	0	0	1	0	1	2
Meyerozyma guilliermondii	1	0	0	0	0	0	1
Pichia cactophila	0	1	0	0	0	0	1
Pichia kudriavzevii/C. krusei	2	4	5	13	11	10	45
Pichia sporocuriosa	0	0	0	0	1	0	1
Saccharomyces cerevisiae	4	9	10	5	3	0	31
Magnusiomyces clavatus	0	0	0	1	0	0	1
Sporopachydermia lactativora	0	1	0	1	0	0	2

More than 90% of candidemia cases are attributed to only five *Candida* species, namely *C. albicans, C. glabrata, C. krusei, C. tropicalis* and *C. parapsilosis* (Parslow and Thornton 2022) with *C. albicans* being the predominant causative agent. In the present work the most isolated species was *C. krusei,* an emerging nosocomial fungal pathogen (Chu et al., 2023; Chamnipa et al., 2018) and is grouped in the medium-risk group of the recently released fungal priority pathogens list (WHO, 2022).

Among the top 5 pathogenic *Candida* species, *C. krusei* is the least well-studied and normally uncommon in the human gut microbiome (Douglass et al., 2018) but widespread in nature. So the abundant presence of *C. krusei* might be due to their frequent occurrence in nature and not necessarily from infected individuals. *C. krusei* can cause significant morbidity and mortality in patients who are immunocompromised (Aslani et al., 2018; Nagarathnamma, et al., 2017) and can cause an array of diseases such as endophthalmitis, and osteomyelitis (Jamiu et al., 2021). It is intrinsically resistant to fluconazole (Morio et al., 2017). There also seems to be a strong genetic relatedness between environmental strains and clinical strains (Douglass et al., 2018), which begs the question if humans are acquiring *C. krusei* infections from the environment or if clinical strains are polluting the environment. A similar conclusion was made by Zulli and colleagues (2024) on the relation between *C. auris* levels in wastewater and their reported incidences, the detected level of *C. auris* does not necessarily match population-level incidence.

Growth study on several yeast isolates

To determine the ability of the various yeast species to grow in the wastewater during transit through the system can contribute to their cell number and likelihood of isolation, the ability of a representative isolate from each species was tested for growth in a pooled wastewater sample. Yeast with similar growth curves were grouped in the following figures (figure 3.3A-D). The isolates in figures 3.3A-C were able to grow in the wastewater sample. In figure 3.3A it can be seen that *Pichia kudriavzevii*/ *C. krusei* grew the best of all the tested strains, reaching an OD_{600} of 0.38 at 24 hours. Interestingly *C. krusei* was the most prevalent species isolated. The same observation can be made for *C. glabrata* (figure 3.3A), the second most isolated species, which grew to a final OD_{600} of 0.27. This could point to their abundant occurrence possibly being linked to their ability to grow well in wastewater. In contrast, *Magnusiomyces clavatus* (previously known as *Geotrichum clavatum* and *Saprochaete clavata*) and *Candida palmioleophila* (figure 3.3A) were not often isolated, although they were also able to grow in wastewater. Although the ecology of *M. clavatus* is not well studied, other *Magnusiomyces* spp. are

ubiquitous in the environment and the digestive tract of animals and humans (Zein et al., 2020). Similarly, *C. palmioleophila* is an environmental yeast with high lipolytic activity, originally isolated from palm oil (Nakase et al. 1988). Interestingly, this yeast was previously isolated from hospital wastewater (Treviñjo-Trejo et al., 2023). This may indicate other factors, including human and environmental sources, that influence the occurrence of these yeasts in wastewater, and not just their ability to grow in this environment.

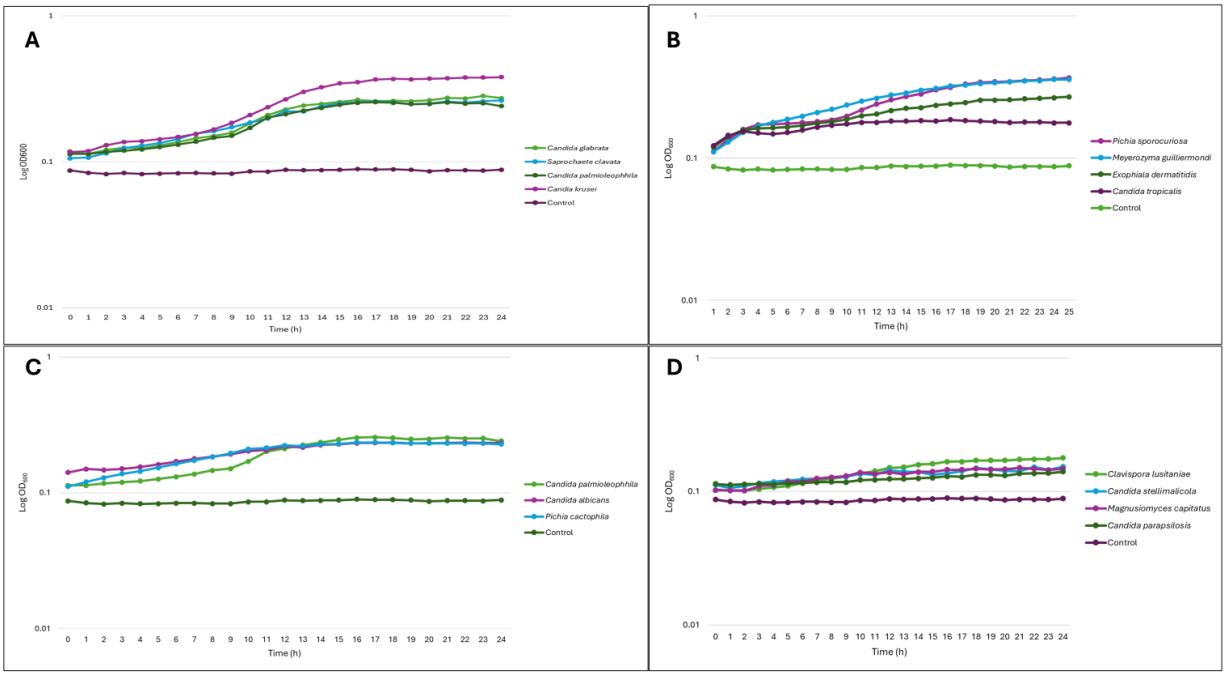


Figure 3.3 Growth of representative yeast isolates. Values represent the average of 3 independent experiments. In all cases, the standard deviations were ≤ 10

Interestingly *Saccharomyces cerevisiae, Hanseniaspora pseudoguilliermondii* and *Sporopachydermina lavtivora* were not able to grow in the wastewater (Figure 3.4) and both *H. pseudoguilliermondii* and *Sporopachydermina lavtivora* were only isolated once, indicating that wastewater is not an ideal habitat for these yeasts. Interestingly, *S. cervisiae* was the third most isolated species. The fact that it seems unable to grow in the wastewater, suggests multiple independent introductions of this yeast to the wastewater system over the 12 month period.

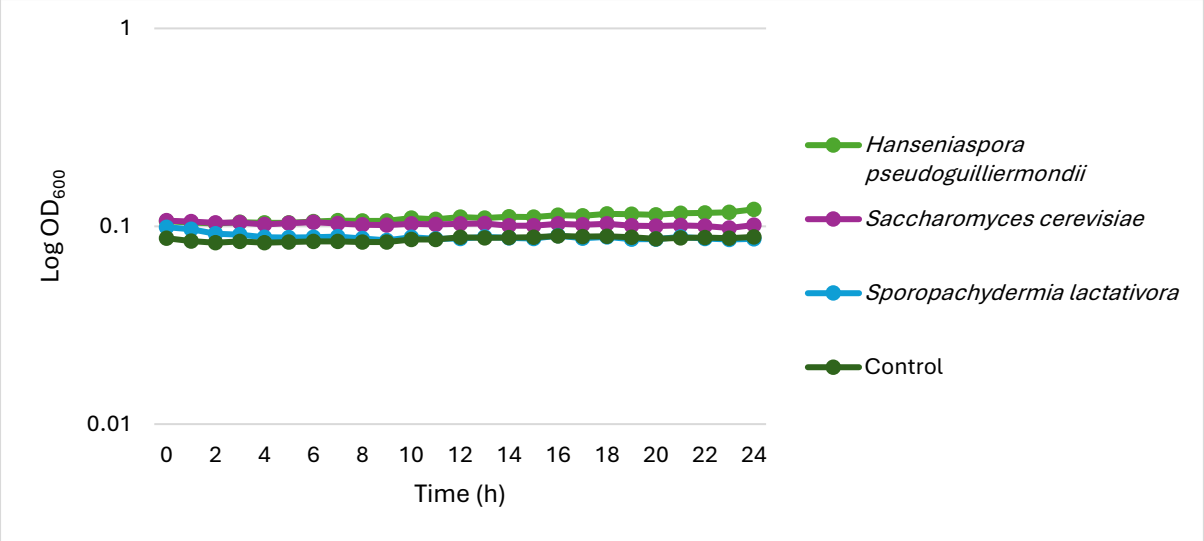


Figure 3.4 Growth of representative yeast isolates. Values represent the average of 3 independent experiments. In all cases, the standard deviations were ≤ 10.

A possible limitation of this experiment is that antifungals were not removed from the wastewater. Samples were filtered to remove bacteria and other macro particulates, but no changes were made to the physio-chemical make-up of the water. So it might be that poor growth is due to the presence of antifungals that inhibit their growth. This will be addressed in the next chapter.

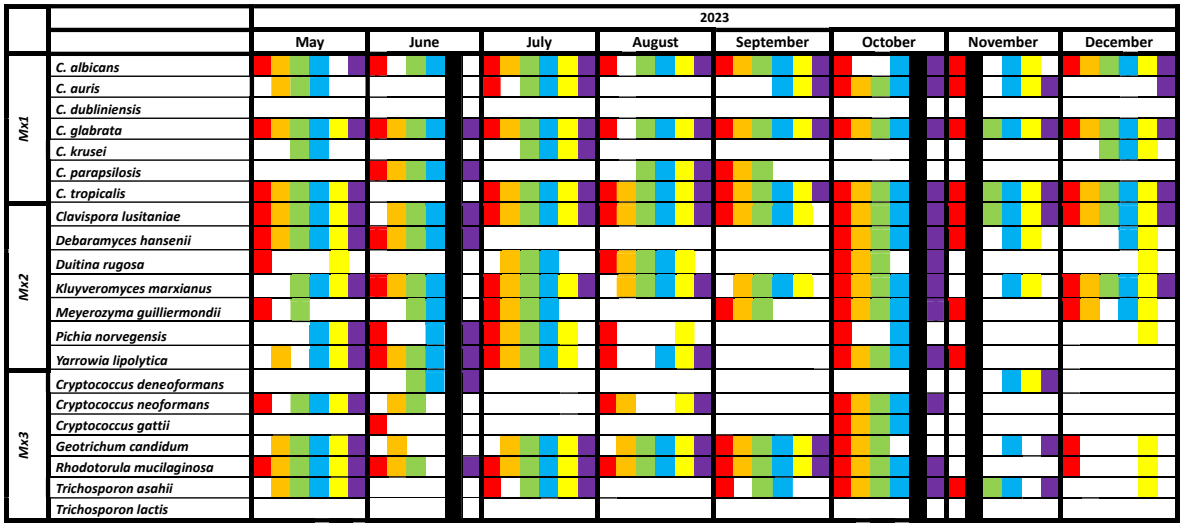
In line with physiochemical parameters, the dissolved oxygen concentration (DOC) was not considered in this study, this may also influence the growth observed for the different yeast isolates. The DOC of wastewater would be greater upon collection than in samples that have been stored for some time. DOC also decreases due to the presence of other components. Although plates were shaken before each optical density measurement to both resuspend cells and allow for oxygen dispersion through the medium, this may not reflect realistic oxygen concentrations in wastewater. Thus, according to the growth study data, some isolates may seem to grow less than others but could grow efficiently in treatment plants.

Culture-independent surveillance identifies pathogenic yeasts

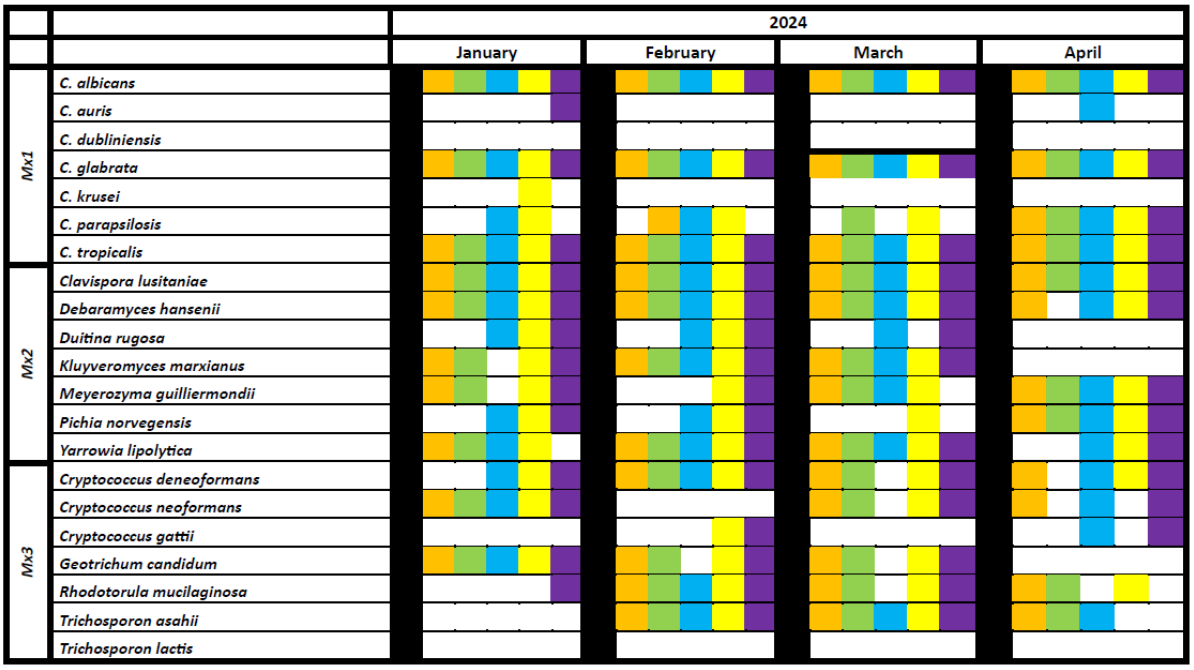
Figures 5 depicts the results for the multiplex PCRs for 2023 and 2024 respectively. During 2023 (Figure 3.5A) frequently detected major *Candida* pathogens were *C. glabrata, C. tropicalis* and *C. albicans*, while *C. auris* was detected in a few samples. *Clavispora lusitaniae, Kluyveromyces marxianus* were most frequently detected as minor *Candida* pathogens and *Geotrichum candidum, Rhodotorula mucilaginosa* and *Trichosporon asahii* as most frequent non-*Candida* pathogens. Similar results were

seen in 2024 except for more frequent detection of *Cryptococcus deneoformans* than in 2023 (Figure 3.5B). Two species, *C. dubliniensis* and *T. lactis* were not detected. Although this may indicate that these yeasts are not present in the wastewater, it is important to also note that the multiplex PCR may not be discriminating enough to detect these two species.

A



B



Colour	Meaning
	University of The Free State
	Bainsvlei treatment works
	Woodland Hills treatment works
	Bloemwater treatment works
	Sterkwater treatment works
	North East treatment works
	No data collected

Figure 3.5 Multiplex PCR results from May 2023 to April 2024

From the multiplex data *C. glabrata* was the most prevalent, occurring in 98.5% of all samples, followed by *Clavispora lusitaniae* (96.9%) and *C. tropicalis* (92.3%) (Figure 3.6).

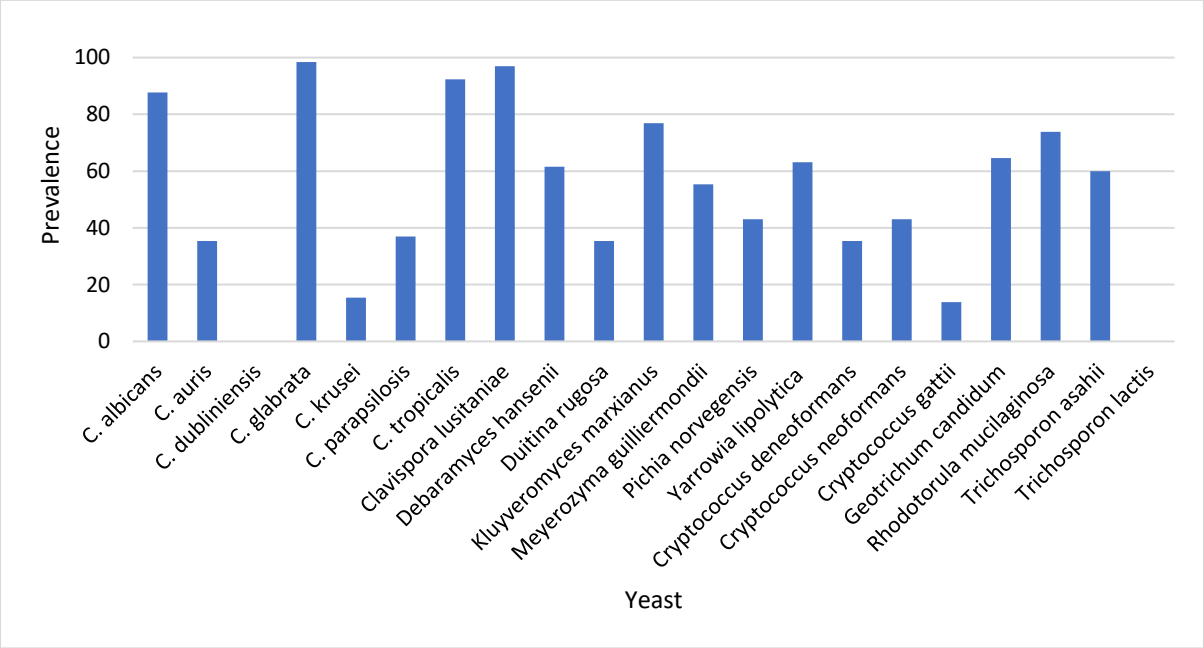


Figure 3.6 Yeast prevalence over 12 months according to the data from the multiplex PCR

It's important to consider that aquatic environments have a low concentration of yeasts when compared to the bacterial, viral and human cells, thus the yeast DNA extracted from samples may only make up a small proportion of the total DNA extracted. This could make molecular detection of specific yeast species difficult since they may occur at too low concentrations. Yet this limitation can be remedied by also using culture-dependent techniques.

Correlation between culture-dependent and independent surveillance

A recently published article (Correa-Moreira et al., 2024) screening wastewater for Sars-CoV-2 and yeasts using polyphasic taxonomy, identified *C. albicans, C. krusei, C. palmioleophila, C. tropicalis* and *C. utilis* to be present. These species were also detected in our study using both multiplex PCR and culture-dependent results, except for *C. utilis*.

Agreement between the culture-dependent and independent data can be seen by the identification of *C. glabrata* as a dominant species in wastewater by both methods. This corresponds with the study of Steffen and colleagues (2023) who found that *C. glabrata* is associated with contamination of water

Interestingly, *C. krusei*, which was the most isolated species, was only detected using multiplex PCR in 15.4% of the samples. In addition, no basidiomycetous yeasts were isolated, although they were detected using the multiplex PCR but were not isolated. We were also unable to isolate *C. auris*, although this yeast was detected by multiplex PCR in 36.4% of samples. This may indicate that the culture-independent technique is more sensitive to species that may occur in low cell numbers in the samples.

Influence of weather on yeast diversity

Meteorological data was collected to investigate the potential influence of environmental conditions on yeast diversity in wastewater (Table 3.4). Bloemfontein falls in a semi-arid climate with hot, wet summers and cold, dry winters. Thus, as can be seen, the dry months are June to August and the wet months are December to February. With the current data, we cannot deduce any trends in the influence of climate/season on yeast diversity. November and March had similar maximum temperatures of (35°C - 36°C), which could explain the higher yeast diversity than the colder months, although the difference is very small.

Table 3.4 Meteorological data recorder over the time sampled.

	Date	Average Temp (week up to day of sampling) °C		Rain (mm)		
		Max	Min	Day before	On the day	Total precipitation for the week up to sample date (mm)
2023	29-May	27	-3.2	3.4	0.6	4.2
	12-June	25	-3.8	0	0	0
	17-July	22	-5.7	0	0	0
	7-August	24	-4.8	0	0	0
	4-September	30	1	0	0	0
	16-October	32	6.2	0.8	3.2	16
	13-November	35	8.7	0	0	12
	4-December	39	7	0	0	0
2024	15-January	33	12	0	0	15
	5-February	33	16	6	0	16
	11-March	36	6.1	0	0	6.4
	15-April	23	3.6	4	5.5	82

In clinical settings, an increase in the number of cases of vaginal candidiasis during the summer time has been reported in Belgium (Donders et al., 2022). These authors identified change in temperature as one of the potential pathophysiological mechanisms that drives this occurrence. Other studies also observed an increase in the number of fungal / yeast skin infections during the summer due to the differences in ambient and skin temperature and humidity (Yalçın et al., 2006; Termorshuizen et al., 2003). An increase in fungal infection may lead to more cells being shed into the wastewater transport system. However, further studies, including estimates of yeast abundance, are needed to indicate the influence of seasonal changes on WBE for pathogenic yeasts.

Association between hospital data and environmental surveillance data

Although the advances made in clinical diagnostics of fungal infections, mainly through the use of molecular techniques and advanced serological methods (White and Price, 2021; Wickes and Wiederhold, 2018) are well known, the public health care sector in South Africa mostly rely on culture-dependant methods to determine the cause of potential fungal infections. This resulted in fungal isolates frequently being unidentified or only identified up to the genus level. Many of the identified fungi were annotated as potential contaminants (possibly at the time of sampling or during cultivation in the laboratory). In many cases, no fungal isolates were obtained from samples from patients with suspected mycoses. This incomplete nature of the data makes the correlation between clinical and wastewater yeasts impossible.

Despite this, in some cases where yeast was cultured and identified from patients, the same species was found in the culture-dependent and independent data, although any causative effect cannot be established. Our detection of *C. auris* during July 2023 is supported by hospital reports of *C. auris* during July (Table S2). This also corresponds to previous studies in which they reported positive clinical cases and positive detection of *C. auris* in wastewater (Barber et al., 2023; Barber et al., 2023; Rossi et al., 2023).

Advantages and limitations of the potential use of WBE for pathogenic yeasts identified in this study

In the few attempts that have been made to detect fungal pathogens in wastewater, only a single yeast has been targeted namely *C. auris* (Zulli et al., 2024; Babler et al., 2023; Barber et al., 2023; Rossi et al., 2023), while detection of multiple pathogenic yeast species in a single sample has only been executed in a laboratory setting on clinical samples (Arastehfar et al., 2020). Various tools, including

multiplex PCR, are available to make the detection of multiple target species in one sample a possibility. By using these tools one would be able to detect multiple target genes in a uniform sample or multiple organisms in a mixed sample in one reaction (Aboutalebian et al., 2021; Mahoney et al., 2007; Persson et al., 2005). The advantages of this system mainly centre around the advantages of a multiplex PCR system over conventional PCR methods. Multiplex PCR provides time-efficient analysis of various samples and is less laborious than conventional PCR or diagnostic tests (Aboutalebian et al., 2021). This creates the opportunity to expand its use as a diagnostic tool and further broaden our knowledge of the transmission and occurrence of pathogenic yeast in communities.

As much promise as this system shows, there are still a few limitations to consider. The study by Babler and colleagues (2023) reported a weak/insignificant correlation between the number of *C.auris* hospital cases and qPCR detection which they potentially attributed either to dilution or the degradation of the target organism by the time of sampling. This is an important point to consider when optimising and interpreting results from WBE for pathogenic yeast. This could be mediated by concentrating samples which allows a large volume of wastewater to be analysed and thus increases the concentration of the target organism (Barber et al., 2023; Rossi et al., 2023). This approach was used in our study where samples were centrifuged and filtered through a 0.45 µm nitrocellulose filter disc before DNA extraction.

From previous studies investigating the application of WBE to pathogenic yeast, the correlation between hospital census on *C. auris* infections and their levels in wastewater is still unclear (Zulli et al., 2024; Babler et al., 2023; Barber et al., 2023; Rossi et al., 2023). Thus, more research comparing the occurrence and levels of pathogenic yeast in wastewater to their occurrence in clinical settings is required to validate wastewater surveillance systems as efficient and accurate community monitoring systems for pathogenic yeast outbreaks. A shortcoming to this surveillance system is that some of the yeast present in wastewater naturally occur in the environment and are not necessarily shed from infected individuals (Zuli et al., 2024), thus care should be taken when interpreting the data collected from these studies. In addition, as we showed, certain yeasts can grow in wastewater and may potentially form more permanent biofilms within the system, which may act as a constant source of these yeasts. This would hinder any time-based correlation between the presence of the yeast in the community and hospital infections. That being said, the presence of *C. glabrata* in wastewater may be used as a potential indicator of fecal contamination of water and should be investigated further.

Conclusions

In this study, we presented the potential application of a wastewater surveillance system specifically for pathogenic yeast species in the Bloemfontein area. It was determined that a wide range of potentially pathogenic yeast species can be found in the influent of wastewater treatment plants with *Candida* species, especially *C. galbrata*, being abundant according to both culture-dependent and i-independent methods. Growth studies indicate that wastewater may act as a hub that promotes yeast growth, yeast species that were most frequently isolated (*C. krusei and C. glabrata*) showed the best growth in wastewater while others that were isolated once showed poor to no growth. However, other factors may play a role in determining what yeast are present in wastewater rather than just their ability to grow in this environment. The multiplex PCR shows a better sensitivity toward detecting cells that may occur at lower concentrations than the culture-dependant methods, this was seen in the frequent detection of *C. auris* using PCR but not being able to isolate this yeast.

The influence of seasonal variations in the occurrence of yeast in wastewater is still unclear, it seems that summer months yield more fungal infections due to warm and humid conditions. This leads to more yeast species being shed into sewer systems than during winter months. Finally, there is a need to use more advanced methods of identification in diagnostics laboratories in the Bloemfontein, Mangaung Metropole area, as the current results from these laboratories are often incomplete and can only be used to speculate correlations between the multiplex PCR and hospital data.

The application of wastewater surveillance to monitor pathogenic yeast is plausible and does show promise although much more research and optimisation of techniques are needed to overcome the limitations presented in this study.

References

Aboutalebian S, Ahmadikia K, Fakhim H, Chabavizadeh J, Okhovat A, Nikaeen M and Mirhendi H (2021) Direct Detection and Identification of the Most Common Bacteria and Fungi Causing Otitis Externa by a Stepwise Multiplex PCR. Front Cell Infect Microbiol 11: 644060. doi: 10.3389/fcimb.2021.644060

Anis E, Kopel E, Singer SR, et al. (2013) Insidious reintroduction of wild poliovirus into Israel. Euro Surveill 18: 20586. doi: 10.2807/1560-7917.ES2013.18.38.20586

Arastehfar A, Carvalho A, Nguyen MH, Hedayati MT, Netea MG, Perlin DS, Hoenigl M (2020) COVID-19-Associated Candidiasis (CAC): An Underestimated Complication in the Absence of Immunological Predispositions? J Fungi 6: 211. doi: https://doi.org/10.3390/jof6040211

Aslani, N., Janbabaei, G., Abastabar, M, Meis JF, Babaeian M, Khodavaisy S, Boekhout T, Badali (2018) Identification of uncommon oral yeasts from cancer patients by MALDI-TOF mass spectrometry. BMC Infect Dis 18: 24. doi: https://doi.org/10.1186/s12879-017-2916-5

Ayanbimpe GM, Abbah VE, Ior CA (2012) Yeasts and yeast-like fungal contaminants of water used for domestic purposes in Jos, Nigeria. Microbiol Res 3: e24. doi: 10.4081/mr.2012.e24

Babič MN, Zalar P, Zenko B, Džeroski S, Gunde-Cimerman N (2016) Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances. Fungal Ecol 20: 30-39. doi: http://dx.doi.org/10.1016/j.funeco.2015.10.001

Babler K, Sharkey M, Arenas S, Amirali A, Beaver C, Comerford S, Goodman K, Grills G, Holung M, Kobetz E, Laine J, Lamar W, Mason C, Pronty D, Reding B, Schürer S, Schaefer Solle N, Stevenson M, Vidović D, Solo-Gabriele H, Shukla B (2023) Detection of the clinically persistent, pathogenic yeast spp. *Candida auris* from hospital and municipal wastewater in Miami-Dade County, Florida. Sci Total Environ 10;898: 165459. doi: 10.1016/j.scitotenv.2023.165459

Barber C, Crank K, Papp K, Innes GK, Schmitz BW, Chavez J, Rossi A, Gerrity D (2023) Community-Scale Wastewater Surveillance of *Candida auris* during an Ongoing Outbreak in Southern Nevada. Environ Sci Technol 57: 1755-1763. doi: 10.1021/acs.est.2c07763

Bleve G, Grieco F, Cozzi G, Logrieco A, Visconti A (2006) Isolation of epithetic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. Int J Food Microbiol 108: 204-209. doi: https://doi.org/10.1016/j.ijfoodmicro.2005.12.004

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and Multi-National Prevalence of Fungal Diseases-Estimate Precision. J Fungi (Basel) 3: 57. doi: 10.3390/jof3040057

Brandão J, Weiskerger C, Valério E, Pitkänen T, Meriläinen P, Avolio L, Heaney CD, Sadowsky MJ (2022) Climate change impacts on microbiota in beach sand and water: looking ahead. Int J Environ Res Public Health 19: 1444. doi: https://doi.org/10.3390/ijerph19031444

Brumfield KD, Leddy M, Usmani M, Cotruvo JA, Tien CT, Dorsey S, Graubics K, Fanelli B, Zhou I, Registe N, Dadlani M (2022) Microbiome analysis for wastewater surveillance during COVID-19. mBio 13: pp.e00591-22. doi: https://doi.org/10.1128/mbio.00591-22

Cassinotti P, Siegl G (1998) A nested-PCR assay for the simultaneous amplification of HSV-1, HSV-2, and HCMV genomes in patients with presumed herpetic CNS infection. J Virol Methods 71**:** 105–114. doi: 10.1016/s0166-0934(97)00203-6

Chamnipa N, Thanonkeo S, Klanrit P, Thanonkeo P (2018) The potential of the newly isolated thermotolerant yeast *Pichia kudriavzevii* RZ8-1 for high-temperature ethanol production. Braz J Microbiol 49: 378-391. doi: 10.1016/j.bjm.2017.09.002

Chen J, Tian S, Han X, Chu Y, Wang Q, Zhou B, Shang H (2020) Is the superbug fungus really so scary? A systematic review and meta-analysis of global epidemiology and mortality of *Candida auris*. BMC Infect Dis 20: 827. doi: 10.1186/s12879-020-05543-0

Chu Y, Li M, Jin J, Dong X, Xu K, Jin L, Qiao Y, Ji H (2023) Advances in the Application of the Non-Conventional Yeast *Pichia kudriavzevii* in Food and Biotechnology Industries. J Fungi 9: 170. doi: https://doi.org/10.3390/jof9020170

Cogliati M, Arikan-Akdagli S, Barac A et al. (2023) Environmental and bioclimatic factors influencing yeasts and molds distribution along European shores. Sci Total Environ 859: 160132. doi: 10.1016/j.scitotenv.2022.160132

Cooke WB, Pipes WO (1969) The occurrence of fungi in activated sludge. Mycopathologia 40: 249–270. doi: https://doi.org/10.1007/BF02051779

Cooke WB (1970) Fungi associated with the activated-sludge process of sewage treatment at the Lebanon, Ohio, sewage treatment plant. Ohio J Sci 70: 129–146

Corrêa-Moreira D, da Costa GL, de Lima Neto RG, *et al.* (2024) Screening of *Candida* spp. in wastewater in Brazil during COVID-19 pandemic: workflow for monitoring fungal pathogens. BMC Biotechnol 24: 43. doi: https://doi.org/10.1186/s12896-024-00868-z

Costa GLd, Negri M, Miranda RPRd, Corrêa-Moreira D, Pinto TCA, Ramos LdS, Ferreira DG, Salomão B, Fumian TM, Mannarino CF, et al. (2023) *Candida palmioleophila*: A New Emerging Threat in Brazil? J Fungi 9: 770. doi: https://doi.org/10.3390/jof9070770

Diamond MB, Keshaviah A, Bento AI et al. (2022) Wastewater surveillance of pathogens can inform public health responses. Nat Med 28: 1992–1995. doi: https://doi.org/10.1038/s41591-022-01940-x

Donders GGG, Ruban K, Donders F, Reybrouck R (2022) Lab-Based Retrospective 10-Year Analysis Shows Seasonal Variation of Vaginal *Candida* Infection Rates in Belgium. J Clin Med 11: 574. doi: 10.3390/jcm11030574

Douglass AP, Offei B, Braun-Galleani S, Coughlan AY, Martos AAR, Ortiz-Merino RA, Byrne KP, Wolfe KH (2018) Population genomics shows no distinction between pathogenic *Candida krusei* and environmental *Pichia kudriavzevii*: One species, four names. PloS Pathog 14: e1007138. doi: https://doi.org/10.1371/journal.ppat.1007138

Egger NB, Kainz K, Schulze A, Bauer MA, Madeo F, Carmona-Gutierrez D (2022) The rise of *Candida auris*: from unique traits to co-infection potential. Microb Cell 9: 141-144. doi: 10.15698/mic2022.08.782

Gangneux JP, Brandao J, Segal E, the ECMM/ISHAM MYCOSANDS study group (2024) Knowledge and regulation on fungal contamination of sand and water: Progress report and perspectives. Med Mycol 62: myad137. doi: https://doi.org/10.1093/mmy/myad137

Harrigan WF, McCance EM (1966) Laboratory Methods in Microbiology. Vol. 54, Academic Press, Cambridge, 970.

Hoenigl M, Seidel D, Sprute R, Cunha C, Oliverio M, Goldman GH, Ibrahim AS, Carvalho A (2022) COVID-19-associated fungal infections. Nat Microbiol *7*: 1127-1140. doi: https://doi.org/10.1038/s41564-022-01172-2

Jamiu AT, Albertyn J, Sebolai OM, Pohl CH (2021) Update on *Candida krusei*, a potential multidrug-resistant pathogen. Med Mycol 59: 14–30. doi: https://doi.org/10.1093/mmy/myaa031

https://www.visualcrossing.com/weather-history/Bloemfontein/metric (accessed 19 February 2024)

Kacprzak M, Neczaj E, Okoniewska E (2005) The comparative mycological analysis of wastewater and sewage sludges from selected wastewater treatment plants. Desalination 185: 363–370. doi: https://doi.org/10.1016/j.desal.2005.03.085

Kainz K, Bauer MA, Madeo F, Carmona-Gutierrez D (2020) Fungal infections in humans: the silent crisis. Microb Cell 7: 143-145. doi: 10.15698/mic2020.06.718

Mahony J, Chong S, Merante F, Yaghoubian S, Sinha T, Lisle C, Janeczko R (2007) Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay. J Clin Micriobiol 45: 2965-2970. doi: https://doi.org/10.1128/jcm.02436-06

McCall C, Wu H, Miyani B, Xagoraraki I (2020) Identification of multiple potential viral diseases in a large urban center using wastewater surveillance. Water Res 184: 116160. doi: https://doi.org/10.1016/j.watres.2020.116160

Medeiros AO, Missagia BS, Brandão LR, Callisto M, Barbosa FAR, Rosa CA (2012) Water quality and diversity of yeast from tropical lakes and rivers from the Rio Doce basin in Southeastern Brazil. Braz J Microbiol 43: 1582-1594. doi: https://doi.org/10.1590/S1517-83822012000400043

Molina F, López-Acedo E, Tabla R, Roa I, Gómez A, Rebollo JE (2015) Improved detection of *Escherichia coli* and coliform bacteria by multiplex PCR. BMC Biotechnol 15: 48. doi: https://doi.org/10.1186/s12896-015-0168-2

Monapathi ME, Horn S, Vogt T, van Wyk D, Mienie C, Ezeokoli OT, Coertze R, Rhode O, Bezuidenhout CC (2021) Antifungal agents, yeast abundance and diversity in surface water: Potential risks to water users. Chemosphere 274: 129718. doi: https://doi.org/10.1016/j.chemosphere.2021.129718

Morio F, Jensen RH, Le Pape P, Arendrup MC (2017) Molecular basis of antifungal drug resistance in yeasts. Int J Antimicrob Agents 50: 599-606. doi: https://doi.org/10.1016/j.ijantimicag.2017.05.012

Moser D, Biere K, Han B, Hoerl M, Schelling G, Choukér A, Woehrle T (2021) COVID-19 Impairs Immune Response to *Candida albicans*. Front Immunol 12: 640644. doi: https://doi.org/10.3389/fimmu.2021.640644

Mukherjee V, Radecka D, Aerts G, Verstrepen KJ, Lievens B, Thevelein JM (2017) Phenotypic landscape of non-conventional yeast species for different stress tolerance traits desirable in bioethanol fermentation. Biotechnol Biofuels 10: 216. doi: 10.1186/s13068-017-0899-5

Munoz P, Giannella M, Fanciulli C, Guinea J, Valerio M, Rojas L, Rodriguez-Creixems M, Bouza E (2011) *Candida tropicalis* fungaemia: incidence, risk factors and mortality in a general hospital. Clin microbiol & infect 17: 1538-1545. doi: 10.1111/j.1469-0691.2010.03338.x

Nagarathnamma T, Chunchanur SK, Rudramurthy SM, Vineetha KR, Ramamurthy K, Joseph J, Ambica R (2017) Outbreak of *Pichia kudriavzevii* fungaemia in a neonatal intensive care unit. J Med Microbiol 66: 1759–1764. doi: https://doi.org/10.1099/jmm.0.000645

Nakase T, Itoh M, Suzuki M, Komagata K, KODAMA T (1988) *Candida palmioleophila* sp. nov., a yeast capable of assimilating crude palm oil, formerly identified as *Torulopsis candida*. J gen appl microbiol 34: 493-498. doi: https://doi.org/10.2323/jgam.34.493

Navarro-Arias MJ, Hernández-Chávez MJ, García-Carnero LC, Amezcua-Hernández DG, Lozoya-Pérez NE, Estrada-Mata E, Mora-Montes HM (2019). Differential recognition of *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris* by human innate immune cells. Infect Drug Resist 12: 783–794. doi: https://doi.org/10.2147/IDR.S197531

Negri M, Silva S, Henriques M. *et al.* (2012) Insights into *Candida tropicalis* nosocomial infections and virulence factors. Eur J Clin Microbiol Infect Dis 31: 1399–1412. doi: https://doi.org/10.1007/s10096-011-1455-z

Parslow BY, Thornton CR (2022) Continuing Shifts in Epidemiology and Antifungal Susceptibility Highlight the Need for Improved Disease Management of Invasive Candidiasis. Microorganisms 10: 1208. doi: 10.3390/microorganisms10061208

Paul JR, Trask JD, Culotta CS (1939) Poliomyelitic virus in sewage. Science 90: 258–259.

Pereira VJ, Basílio MC, Fernandes D, Domingues M, Paiva JM, Benoliel MJ, Crespo MT, San Romão MV (2009) Occurrence of filamentous fungi and yeasts in three different drinking water sources. Water Res 43: 3813–3819. doi: https://doi.org/10.1016/j.watres.2009.05.024

Persson K, Hamby K, Ugozzoli LA (2005) Four-color multiplex reverse transcription polymerase chain reaction—Overcoming its limitations. Anal Biochem 344: 33-42. doi: 10.1016/j.ab.2005.06.026

Pour AH, Salari S, Almani PGN (2018) Oropharyngeal candidiasis in HIV/AIDS patients and non-HIV subjects in the Southeast of Iran. Curr Med Mycol 4: 1-6. doi:10.18502/cmm.4.4.379

Prigitano A, Trovato L, Esposto MC et al. (2023) Fungal diversity in lake and sea beaches of Italy: relevance to human health. Sci Total Environ 859: 160417. doi: 10.1016/j.scitotenv.2022.160417

Reyneke B, Ndlovu T, Khan S, Khan W (2017) Comparison of EMA-, PMA- and DNase qPCR for the determination of microbial cell viability. Appl Microbiol Biotechnol 101: 7371–7383. doi: 10.1007/s00253-017-8471-6

Rossi A, Chavez J, Iverson T, Hergert J, Oakeson K, LaCross N, Njoku C, Gorzalski A, Gerrity D (2023) *Candida auris* Discovery through Community Wastewater Surveillance during Healthcare Outbreak, Nevada, USA, 2022. Emerg Infect Dis 29: 422-425. doi: 10.3201/eid2902.221523

Steffen HC, Smith K, van Deventer C, Weiskerger C, Bosch C, Brandão J, Wolfaardt G, Botha A (2023) Health risk posed by direct ingestion of yeasts from polluted river water. Water Res 231: 119599. doi: https://doi.org/10.1016/j.watres.2023.119599

Taniuchi M, Verweij JJ, Noor Z, Sobuz SU, van Lieshout L, Petri Jr. WA, Haque R, Houpt ER (2011) High Throughput Multiplex PCR and Probe-based Detection with Luminex Beads for Seven Intestinal Parasites. Am J Trop Med Hyg. 84: 332–337. doi:10.4269/ajtmh.2011.10-0461

Termorshuizen, F., Hogewoning, A.A., Bouwes Bavinck, J.N., Goettsch, W.G., De Fijter, J.W. and Van Loveren, H., 2003. Skin infections in renal transplant recipients and the relation with solar ultraviolet radiation. Clin transplant 17: 522-527. doi: https://doi.org/10.1046/j.0902-0063.2003.00099.x

Tiwari S, Dhole TN (2018) Assessment of enteroviruses from sewage water and clinical samples during eradication phase of polio in North India. Virol J 15: 157. doi: https://doi.org/10.1186/s12985-018-1075-7

Treviño-Trejo AA, Vázquez-Leyva S, Vallejo-Castillo L, Macías-Palacios Z, Pérez-Tapia SM, Cruz-Maya JA, Jan-Roblero J (2023) *Candida palmioleophila* Carba14 is capable of degrading carbamazepine. Bioremediat J 28: 191–201. doi: https://doi.org/10.1080/10889868.2023.2268783

White LP, Price JS (2021) Recent Advances and Novel Approaches in Laboratory-Based Diagnostic Mycology. J Fungi 7: 41. doi: https://doi.org/10.3390/ jof7010041

WHO (2022) WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization

Wickes BL, Wiederhold NP (2028) Molecular diagnostics in medical mycology. Nat Commun 9: 5135. doi: https://doi.org/10.1038/s41467-018-07556-5

Wise J (2022) Poliovirus is detected in sewage from north and east London. BMJ 377 doi: https://doi.org/10.1136/bmj.o1546

Wu F, Xiao A, Zhang J (2021) Wastewater surveillance of SARS-CoV-2 across 40 U.S. states from February to June 2020. Water Res 202: 117400. doi: https://doi.org/10.1016/j.watres.2021.117400

Yalçın B, Tamer E, Toy GG, Öztaş P, Hayran M, Allı N (2006) The prevalence of skin diseases in the elderly: analysis of 4099 geriatric patients. Int J Dermatol 45: 672-676. doi: https://doi.org/10.1111/j.1365-4632.2005.02607.x

Yamaguchi MU, Rampazzo RCP, Yamada-Ogatta SF, Nakamura CV, Ueda-Nakamura T, Filho BPD (2007) Yeasts and Filamentous Fungi in Bottled Mineral Water and Tap Water from Municipal Supplies. Braz Arch Biol Technol 50: 1-9. doi: https://doi.org/10.1590/S1516-89132007000100001

Yang Q, Zhang H, Li X, Wang Z, Xu Y, Ren S, Chen X, Xu Y, Hao H, Wang H (2013) Extracellular enzyme production and phylogenetic distribution of yeasts in wastewater treatment systems. Bioresour Technol 129: 264-273. doi: https://doi.org/10.1016/j.biortech.2012.11.101

Zein SEI, Hindy JR, Kanj SS (2020) Invasive *Saprochaete* Infections: An Emerging Threat to Immunocompromised Patients. Pathogens 9: 11: 922. doi: https://doi.org/10.3390/pathogens9110922

Zulli A, Chan EMG, Shelden B, Duong D, Xu X-RS, White BJ, Wolfe MK, Boehm AB (2024) Prospective study of *Candida auris* nucleic acids in wastewater solids in 190 wastewater treatment plants in the United States suggests widespread occurrence. mBio 15: e0090824. doi: 10.1128/mbio.00908-24

Chapter 4:

Wastewater: A potential source of resistant yeast

(Parts of this chapter have been submitted for publication)

Baker T, Bester PA, Sebolai OM, Albertyn J, Pohl CH (2025) Culture-Dependentand - Independent Wastewater Surveillance for Multiple Pathogenic Yeasts. JFungi 11: 86. doi: https://doi.org/10.3390/ jof11020086

Abstract

Antifungal resistance is a growing concern, and it is imperative that we not only understand the mechanisms of resistance found in yeast and how to combat this, but also where resistance development originates and which environmental factors contribute to resistance development. Other than the clinical environment where patients with infections are exposed to antifungals, the natural environment (aquatic and terrestrial) may also be a potential hub for resistance development. Wastewater treatment plants are recognised as environments contributing to antibiotic resistance in bacteria but only a few studies have investigated their possible contribution to antifungal resistance in pathogenic yeasts. In this study, we aim to investigate this. The concentration of a commonly used azole, fluconazole, was determined using LC/MS. Varying fluconazole levels were detected over 12 months with season potentially influencing concentrations. Calculations to determine the risk for resistance selection of yeast towards fluconazole were done along with microbroth dilutions to investigate resistance or susceptibility of yeast found in wastewater influent. Microbroth dilutions were performed on isolates from months with the lowest and highest fluconazole concentrations to determine their susceptibility/ resistance to fluconazole. *C. krusei* was frequently resistant to fluconazole with *C. albicans* and *C. tropicalis* being susceptible and *C. glabrata* showed dose-dependent susceptibility and one resistant strain. Considering all the data, wastewater seems to support yeast proliferation which is cause for concern when treatment plants are not working, and water remains stagnant for long periods. Fluconazole's presence in wastewater seem to select resistant yeast species while also supporting the growth of susceptible dose-dependent isolates which might develop acquired resistance to fluconazole in this environment.

Keywords: Antifungal-resistance, *C. krusei*, fluconazole, LC/MS.

Introduction

Pathogenic fungi can cause severe to fatal infections in immune-compromised or suppressed individuals (Parslow and Thornton 2022; Perlin et al., 2017), resulting in a mortality rate that exceeds that of malaria and tuberculosis (Bongomin et al., 2017). These numbers continue to increase as the at-risk or susceptible population continues to grow due to the increase in cases of cancer, asthma, HIV/AIDS, chronic obstructive pulmonary disease, tuberculosis (Pour et al.,2018; Bongomin et al., 2017), the Covid-19 pandemic (Moser et al., 2021) and the use of various therapeutic approaches (Alcazar-Fuoli and Mellado, 2014).

Current statistics highlight the seriousness of infections caused by pathogenic fungi, especially infections caused by species within the *Candida* genus. In the U.S. alone, yeast infections rank among the top three causes of bloodstream infections and fourth for all healthcare-associated infections. In these cases, *Candida* spp. were the most frequent culprits (Menu et al., 2023). Invasive candidiasis is the most common form of invasive fungal infection with an estimated global occurrence of 750,000 cases annually, while other pathogenic yeast such as *Cryptococcus* has an annual incidence occurrence of 225 000 cases (Parslow and Thornton, 2022). Thus, effective and efficient treatment is crucial to ensure a decrease in mortality rates. However, this may pose a greater challenge than expected, due to the limited available antifungal agents (Bouz and Doležal, 2021) and the development of antifungal-resistant strains.

To add fuel to the fire, antifungal resistance is increasing in incidence with more frequent identification of antifungal-resistant or tolerant strains. In addition, certain pathogenic yeast, such as *Candida auris* are drug resistant, with certain strains resistant to all treatment options (Chaabane et al., 2019). In addition, others, such as *C. glabrata* show variation in susceptibility towards certain drugs (Duggan and Usher, 2023). This is a global concern due to its widespread occurrence (Berman and Krysan, 2020; Fisher et al., 2018). Current antifungal drugs can be grouped into five classes namely, polyenes, azoles, echinocandins, pyrimidine analogues (Sanglard 2016) and allyamines (Vanreppelen et al., 2023) each targeting specific pathways or membrane components to elicit their fungicidal or static effect. The increase in resistance of fungal species may be attributed to the development of acquired resistance to various antifungal drugs found in their immediate environment including clinical, terrestrial and aquatic environments (Revie et al., 2018).

Numerous studies report the presence of both potentially pathogenic fungal spp. (Medeiros et al., 2012; Yang et al., 2011; Arvanitidou et al., 1999; Yamasato et al., 1974) as well as antifungal agents

(Monaphathi et al., 2021; Assress et al., 2020; Monapathi et al., 2017; Chen et al., 2014) in water. Azoles are the most frequently prescribed drug for superficial and invasive fungal infections (Vanreppelen et al., 2023; Sanglard 2016) and are also frequently used in agricultural fungicides (Li et al., 2019; Toda et al., 2019), thus it is not surprising to find this antifungal as a common pollutant in water sources (Liu et al., 2016; Yao et al., 2016). Other sources of antifungal pollution include pharmaceutical and veterinary runoff, and their use in personal care products (Liu et al., 2016). It is speculated that the close association between antifungals and yeast in different environments, not just in clinical settings, may contribute to the development of antifungal resistance in pathogenic yeast species.

This chapter aims to investigate the levels of fluconazole in wastewater and if the association between this antifungal, and yeasts found in wastewater may influence their susceptibility towards fluconazole.

Materials and methods

Sample collection

Samples were collected from six different sites as indicated in Chapter 3, according to the protocol outlined by the South African Medical Research Council (SAMRC) (SAMRC, 2021). One liter of wastewater influent was collected after the first grit screen, this helps to reduce the number of unwanted particles and contaminants such as toilet paper plastics, insects ect. in the samples. Sampling was done once a month from May 2023 and ending in April 2024 at six different sampling points in Bloemfontein, Mangaung Metro, South Africa. Samples were collected using a bucket or bottle scoop and were stored in closed, labelled containers during transport and kept in a 4°C refrigerator until use, which was approximately two hours after the last sample was collected.

Azole quantification

Wastewater (500 ml) was filtered using glass fibre filter disks (Schleicher and Schuell Bioscience, New Hampshire). Filtrate was then eluted using a Phenomenex Strata ® C18-E (55µm, 70Å) solid phase extraction column (Phenomenex, California USA). Atrazine D3 (40 µl)was used as an internal standard with commercially available fluconazole as an external standard. Columns were washed with 6 ml methanol, followed by 6 ml molecular grade water. Samples were added to through the columns with the aid of a vacuum pump. After thoroughly drying the columns, the fluconazole was eluted using 2 ml methanol followed by 2 ml ethyl acetate. The eluent was dried in a SpeedyVac and reconstituted in 500 µl of a 0.1% formic acid solution.

Samples were analysed using an ABSCIEX 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu HPLC stack as a front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software. Twenty microliter of each extracted sample was separated on a C18 (150mm x 4.6mm, Gemini NX, Phenomenex) column at a flow rate of 300 uL/min using a 3 min gradient from, in positive ionisation mode, 5% solvent A (H_2O/0.1% formic acid) to 95% solvent B (MeOH/0.1% formic acid). Eluting analytes were ionised by electrospray in the TurboV ion source with 500^oC heater temperature to evaporate excess solvent, 30 psi nebuliser gas, 30 psi heater gas and 20 psi curtain gas. The ion spray voltage was set at 5500 V.

A calibration curve was generated for each analyte ranging in concentration from 10 ppm (part per million; mg/L) to 0.0001 ppm with a linear fit through the origin producing a correlation coefficient (r value) in excess of 0.98.

The limit of quantitation (LOQ) of each analyte was determined to be the lowest concentration of the analyte in the calibration curve where the instrument response with a signal to noise (S/N) ratio >10.

The targeted analyses of fluconazole was performed using 3 MRM (multiple reaction monitoring) transitions. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the second and third transitions are used as a qualifier. The qualifiers serves as an additional level of confirmation for the presence of the analyte, the retention time for these two transitions needs to be the same. The peak areas of the unknown samples were related back to a quantified value using the calibration curve for each analyte, normalised to the peak area of the internal standard.

Table 4.1 Mass of the first and third qualifiers for each analyte. The second qualifier was excluded because it acts as the collision cell to produce the third qualifier

Q1 mass	**Q3 mass**	**Analyte**
221.1	179.0	Atrazine-d5 (internal standard)
307.1	220.1	Fluconazole 1
307.1	238.1	Fluconazole 2
307.1	169.1	Fluconazole 3

Risk for resistance selection towards fluconazole

Risk assessment calculations (RQ) were done according to the article by Assress and colleagues (2020). The potential ecological risk posed by fluconazole in wastewater influent was calculated using the risk quotient method (Sousa et al., 2029). In summary, RQ was calculated using the ratio of measured environmental concentration (MEC) and predicted no effect concentration (PNEC) of fluconazole, (equation 1).

$$RQ = \frac{MEC}{PNEC} \quad (1)$$

Equation one was used to calculate the ecological risk of fluconazole resistance selection by fungal species. In this case, the PNEC used was the antifungal concentration that was predicted not to result in the development of drug resistance. PNEC values for the selected antifungal were obtained from

Bengtsson-Palme and Larsson (2016). RQ values were classified as high risk (RQ≥1), medium risk (0.1≤ RQ < 1) and low risk (0.01 ≤RQ < 0.1) as determined by Hanna and colleagues (2018).

Fluconazole susceptibility testing

The methodology presented in this section was adapted from the EUCAST protocol by Rodriguez-Tudela et al. (2008). Yeast isolates obtained during July 2023 and January 2024, as well as the quality control strain, *C. parapsilosis* ATCC 22019, were cultured overnight on yeast malt extract agar (3g/L malt extract, 3g/L yeast extract, 5g/L peptone powder, 10g/L D-Glucose, 16g/L bacteriological agar) at 37°C. Isolates were then standardised in sterile water at a wavelength of 562nm using a reading of 0.5xMcfarland standard as specified in the protocol. The cell concentration after standadisation was 1-5 x 10^6 CFU/ml. An inoculum of 1 ml was concentrated (7830 rpm for 10 min, Centrifuge 5430R Eppendorf® USA: F – 35 – 6 – 30 rotor), the pellet was resuspended in 5 ml filter sterilised 2xRPMI-1640 2% G broth (20.8g/L RPMI1640 powder-without bicarbonate, 60.06g/L MOPS buffer, 36g/L glucose). Aliquots of 50 µl of this inoculum was dispensed into a 96-well plate containing 50 µl of diluted fluconazole (final cell concentration of 1-2 x 10^5 CFU/ml). Fluconazole was prepared in a 2-fold serial dilution ranging between 64 mg/L and 0.125 mg/L. Untreated and cell free controls were included. The plates were incubated at 37°C for 24 h, after which the absorbance was measured at 562 nm.

Results and discussions

Azole quantification

Wastewater is a mixture of compounds which includes pharmaceuticals. Of particular interest in this study was the pharmaceutical product, fluconazole, which is an antifungal used to treat topical to systemic infections caused by several fungal genera. Fluconazole forms part of the azole antifungal group which is frequently used due to the ease of oral administration and cost-effective production. Figure 4.1 indicates the concentration (ng/L) of fluconazole detected at each sample site over the 12 month study period. The highest concentration observed was 95.8 ng/L for Bainsvlei (BV) in December 2023 and Sterkwater (SW) in March 2024. New North East (NNE) wastewater treatment plant had the highest average concentration of fluconazole at 37.9 ng/L for the 12 months. The UFS sample site (which is a raw sewage point) frequently showed low concentration of fluconazole. Although no data is available for UFS from January to April 2024, due to construction taking place obstructing the access

point to the manhole used, this lower trend may indicate that wastewater treatment plants could act as concentrators of fluconazole, similar to what is observed for other pharmaceuticals (Munzhelele et al., 2024). One explanation for the high concentration at NNE might be the diverse group of individuals that contribute to this catchment area. This site is not exclusive to only Bloemfontein residents but also to national and international travelers.

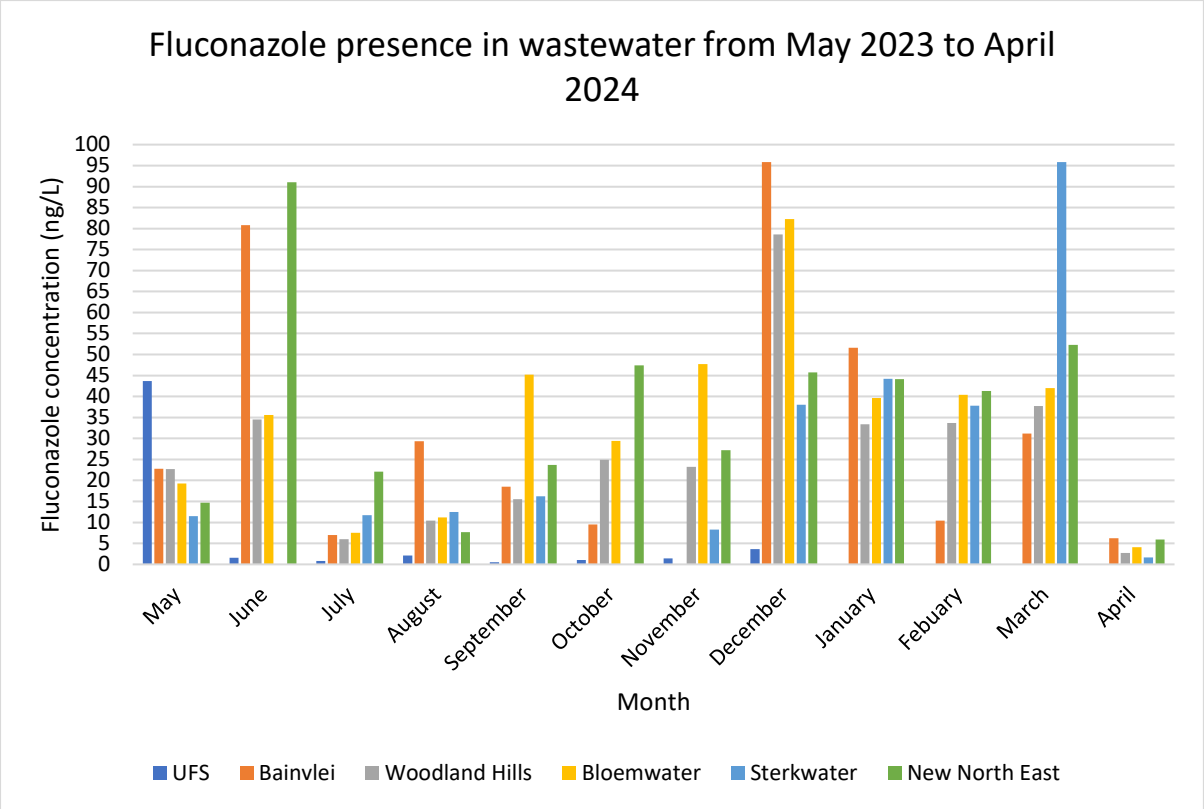


Figure 4.1 Fluconazole presence in wastewater from May 2023 to April 2024. Fluconazole (ng/L) was measured at six different sampling locations using LC/MS

Based on this data, the average and total fluconazole concentrations were calculated for the same period (Figure 4.2). As can be seen, the total fluconazole concentration of all the wastewater sites ranged from 20.6 ng/L (April 2024) to 344 ng/L (December 2023), while the average fluconazole concentration for each month was below 60 ng/L.

Previous studies record fluconazole levels ranging from 178 ng/L to 271 ngl L in freshwater with a few samples with concentrations below level of detection (15ng/L) (Monapahti et al., 2021a). Studies done in Gauteng, South Africa, found fluconazole to be present at a maximum concentration of 302.38 ng/L in 2019 and 9959 ng/L in 2020 in wastewater influent (Assress et al., 2020, Assress et al., 2019). In

Zürich, wastewater influent from several treatment plants measured 32-109 ng/L (Kahle et al., 2008). Wastewater influent in eight urban Romanian wastewater treatment plants measured 49.3-76.8 ng/L (Iancu et al., 2024). Thus, the fluconazole concentrations measured in our study correspond to levels measured in Zürich and Romania, however, in comparison to other South African studies we measured a lower concentration of fluconazole per site. This may be due to lower population density in Mangaung Metro compared to cities in Gauteng.

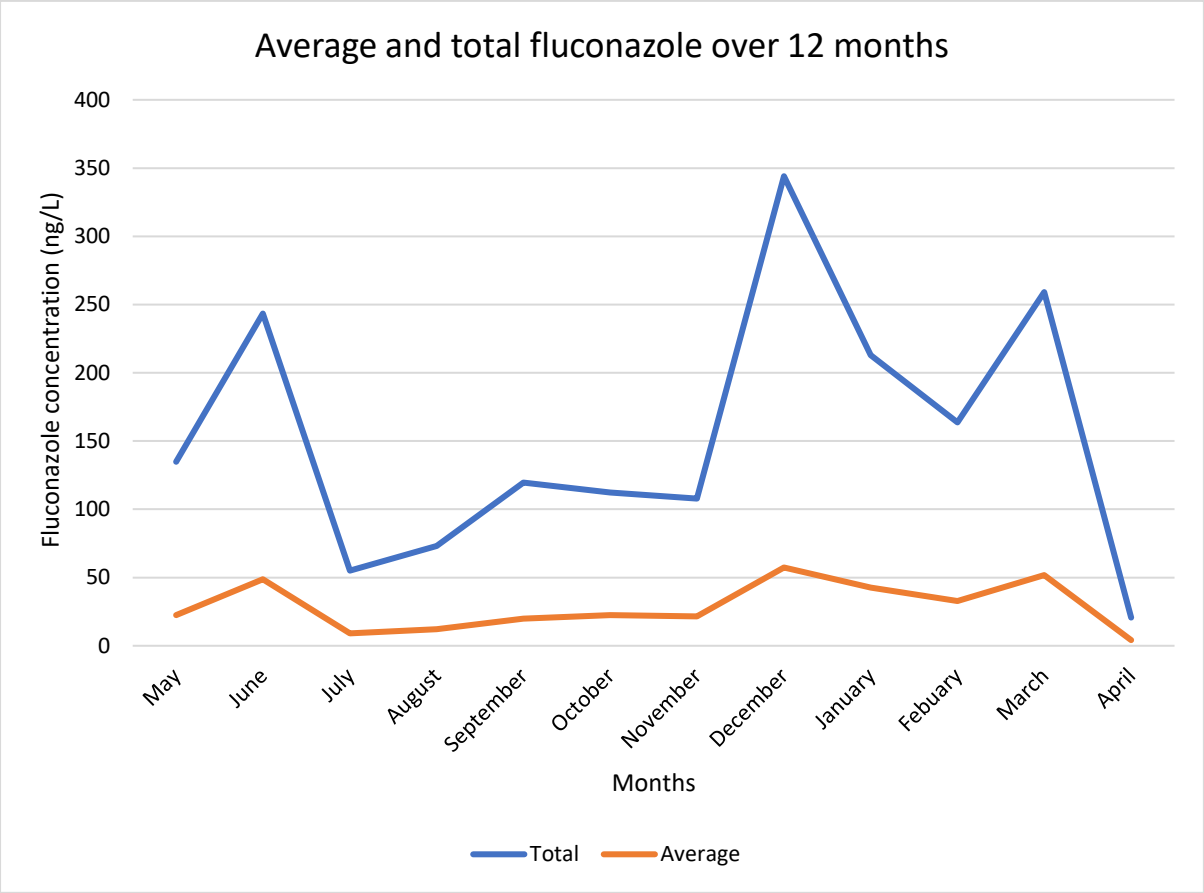


Figure 4.2 Average and total fluconazole (ng/L) measured from May 2023 to April 2024. Average fluconazole concentrations were calculated from all the WWTPs plants for the specific month and the total fluconazole concentrations were calculated by adding together the measured concentrations for all the WWTPs for the specific month

This data also shows the potential influence different seasons might have on the concentrations of fluconazole in wastewater. It seems that there was higher total concentration of fluconazole during

the summer months of South Africa than in the winter months. Other studies have also made this observation (Peng et al., 2012). This is potentially due to the drier climate which is unfavourable for fungal growth, thus, reducing antifungal use during drier months.

Risk for resistance selection towards fluconazole

The PNEC value for fluconazole was 250ng L^{-1}, as determine by Bengtsson-Palme and Larsson (2016). Results (table 4.2) indicate a low to medium risk for resistance selection of fungal species. Medium risk was mostly seen during the summer months, compared to the low associated risk observed during winter months. Measured environmental concentrations (MEC) can be found in table S1 in the supplementary data.

Table 4.2 RQ for fluconazole resistance selection of fungi over 12 months. RQ values were classified as high risk (RQ≥1), medium risk (0.1≤RQ < 1) and low risk (0.01 ≤RQ < 0.1)

	UFS	**BV**	**WL**	**BW**	**SW**	**NNE**	**Total RQ**	**Average RQ**	**RQ classification**
May	0.17	0.09	0.09	0.08	0.05	0.06	0.54	0.09	*LR
June	0.01	0.32	0.14	0.14	0.00	0.36	0.97	0.16	#MR
July	0.00	0.03	0.02	0.03	0.05	0.09	0.22	0.04	LR
August	0.01	0.12	0.04	0.04	0.05	0.03	0.29	0.05	LR
September	0.00	0.07	0.06	0.18	0.06	0.09	0.48	0.08	LR
October	0.00	0.04	0.10	0.12	0.00	0.19	0.45	0.07	LR
November	0.01	0.00	0.09	0.19	0.03	0.11	0.43	0.07	LR
December	0.01	0.38	0.31	0.33	0.15	0.18	1.38	0.23	MR
January	0.00	0.21	0.13	0.16	0.18	0.18	0.85	0.14	MR
February	0.00	0.04	0.13	0.16	0.15	0.17	0.65	0.11	MR
March	0.00	0.12	0.15	0.17	0.38	0.21	1.04	0.17	MR
April	0.00	0.03	0.01	0.02	0.01	0.02	0.08	0.01	LR
Average RQ per site	0.02	0.12	0.11	0.13	0.09	0.14			
RQ classification	LR	MR	MR	MR	LR	MR			

*LR = Low risk

#MR = Medium risk

Fungal communities found in wastewater influent thus stand mostly a medium risk to develop antifungal resistance towards fluconazole. This was especially observed during the summer months with a low risk observed during the winter months. This further supports our hypothesis that wastewater acts as a hub for pathogenic yeast proliferation and might well be contributing to an environment more favourable for antifungal resistance development. However, more studies on this topic are needed to confirm this.

Yeast fluconazole susceptibility testing

To determine if fluconazole concentration in the wastewater influences the drug susceptibility of the yeasts, species with multiple isolates obtained during July 2023 (with relatively low fluconazole levels detected in the wastewater) and March 2024 (with relatively high fluconazole levels in the wastewater) were tested for their susceptibility towards fluconazole. The detailed graphs indicating the responses of all the strains are provided in the supplementary materials. Yeast displaying similar profiles were grouped together in graphs (figures S2A – K). The quality control yeast (*C. parapsilosis* ATCC 22019) also yielded the expected results verifying the accuracy of the results.

Due to the fungistatic nature of fluconazole, MIC values were defined as the lowest concentration with significant (≥50%) growth reduction, relative to the control (Pfaller et al., 2015). When comparing the MIC_{50} of fluconazole for the *C. albicans*, *C. glabrata, C. krusei* and *C. tropicalis* isolates (Table 4.3) to the EUCAST Standards (Arendrup et al., 2020) document, *C. albicans* isolates were susceptible. Most *C. glabrata* isolates were susceptible dose-dependent with one strain (SW11.1) being resistant, while all the *C. tropicalis* isolates were susceptible. As expected, *C. krusei* isolates exhibited very high MIC_{50} values due to this species intrinsic resistance towards fluconazole (EUCAST, 2008). We should also consider that *C. krusei* is often reported as multidrug-resistant, causing serious concern in the clinical field (Jamiu et al., 2021). Interestingly in March 2023 a higher number of *C. krusei* isolates were obtained, which may indicate that the higher fluconazole concentration in the wastewater, may be selected for this resistant yeast species. This is possible since the yeasts could have been introduced into the wastewater system from the natural environment, such as from agricultural run-off or patients with active infections shedding yeast into sewer systems.

Saccharomyces cerevisiae can also act as an opportunistic pathogen, causing fungemia in immunocompromised or critically ill patients (Atıcı et al., 2017; Muñoz et al., 2005). The preferred

treatment is with Amphotericin B and fluconazole (Muñoz et al., 2005). The MIC breakpoints for *S. cerevisiae* have not yet been defined, but following the discussion in an article by Enache-Angoulvant and Hennequin (2005) the MIC_{90} of fluconazole for this yeast is between 16-32 mg/L. In our study the highest required concentration to reach an MIC_{50} was 32mg/L and the lowest was 2 mg/L fluconazole.

Table 4.3 Minimum inhibitory concentrations (MIC) of fluconazole for yeast isolates

Strain name and number	**July 2023***			**Strain name and number**	**March 2024#**		
	MIC_{50} (µg/ml)	**MIC_{90} (µg/ml)**	**MIC (µg/ml)**		**MIC_{50} (µg/ml)**	**MIC_{90} (µg/ml)**	**MIC (µg/ml)**
C. albicans				*C. albicans*			
UFS2.2	<0.125	<0.125	1	WL11.2	<0.125	1	nd
WL2.1	<0.125	nd	nd	WL11.3	<0.125	0.25	8
WL2.5	0.5	nd	nd	WL11.4	0.25	nd	nd
C. glabrata				*C. glabrata*			
BV2.4	2	32	nd	BV1.2	4	nd	nd
BV2.5	8	nd	nd	BV11.4	8	nd	nd
BV2.6	1	nd	nd	B11.6	4	nd	nd
BW2.1	2	nd	nd	NNE11.1	2	nd	nd
BW2.5	4	nd	nd	NNE11.2	4	nd	nd
NNE2.2	4	nd	nd	NNE11.3	4	nd	nd
				SW11.1	64	nd	nd
				SW11.5	4	nd	nd
				WL11.5	4	nd	nd
C. krusei				*C. krusei*			
NNE2.1	64	nd	nd	BV11.3	64	nd	nd
NNE2.5	64	nd	nd	BV11.5	64	nd	nd
NNE2.6	32	64	64	BW11.1	32	64	64
				BW11.6	>64	nd	nd
				NNE11.4	64	nd	nd
				NNE11.5	16	64	64
				SW11.4	16	nd	nd
				SW11.5	32	64	64
C. tropicalis				*C. tropicalis*			
BV21.	<0.125	0.25	0.5	not isolated			
BW2.3	0.5	nd	nd				
UFS2.1	<0.125	16	nd				
S. cerevisiae				*S. cerevisiae*			
BV2.3	8	nd	nd	not isolated			
BW2.2	32	nd	nd				
BW2.4	32	nd	nd				
BW2.6	2	16	32				
UFS2.3	16	nd	nd				
UFS2.4	8	32	nd				
UFS2.5	8	32	nd				
UFS2.6	4	32	nd				
WL2.3	4	nd	nd				

*Average fluconazole concentration in wastewater = 9.2 ng/L

Average fluconazole concentration in wastewater = 57.3 ng/L

nd = MIC90 or MIC ≥64 µg/ml

Clinical settings often document antimicrobial resistance (AMR) but are not the only place this phenomenon can occur. Other settings gaining recognition for the dissemination and evolution of AMR include the natural environment. Most studies investigating how the environment contributes to AMR focus on bacteria whereas few studies investigate this phenomenon in fungi (Stevenson et al., 2022).

In the case of bacteria, the proximity of some of these pathogens allows for the exchange of AMR genes, whereas this is not the case in fungi and other mechanisms of acquired resistance is more likely.

Acquired resistance to antifungals can be achieved by prolonged exposure to antifungals in both clinical and agricultural settings (Snelders et al., 2008). Typical mechanisms of acquired resistance include overexpression of drug targets, alteration of the drug target through amino acid substitution, upregulation of efflux pumps or changes to the cellular pathways governed by the antifungal target (Revie et al., 2018).

From the results we can see that the isolates from March 2024 (high fluconazole concentrations) had overall higher MIC_{50} concentrations than those from July 2023 (low fluconazole concentrations). A previous study investigating the antifungal resistance of yeast in wastewater in the Northwest province, South Africa, they found a positive correlation between fluconazole concentration and resistance of yeast isolated from the same environment, indicating the isolates showed less susceptibility towards fluconazole (Monapahti et al., 2021a; Monapahti et al., 2017). In the study by Monapahti and colleagues (2021a) they reported higher concentrations of fluconazole than in our study which may explain why they saw a positive correlation between fluconazole concentration and resistance. This may support the hypothesis that environmental pollution by fluconazole may contribute to resistance development, although, as mentioned above, an alternative explanation could be that the higher fluconazole concentrations may select for more resistant strains.

Conclusions

In conclusion, fluconazole concentrations in wastewater were within the range reported by other studies. In addition, the concentration varied from month to month and might be influenced by the season (temperature and rainfall), with higher concentrations observed during the summer months than in the winter months. Risk assessment calculations also concluded that during summer months, yeast stand a moderate risk of developing resistance towards fluconazole while low risk occurs during

the winter months. Wastewater treatment plants may also act as a hub for the development of acquired resistance to fluconazole which is seen in the higher number of resistant strains isolated from the month in which the highest concentration of fluconazole was measured, yet more studies are needed to confirm this. However, a possible alternative hypothesis is that fluconazole concentration in the wastewater may select for already resistant strains. This is possible since the yeasts could have been introduced into the wastewater system from the natural environment, such as from agricultural run-off or patients with active infections shedding yeast into sewer systems. Regardless of how the resistant strains arrive in the wastewater, they may pose serious health risks to susceptible persons that come into contact with this water, as part of their work or due to poor infrastructure leading to sewage spills. This chapter also supports the notion that novel antifungals are of critical concern.

References

Alcazar-Fuoli L and Mellado E (2014) Current status of antifungal resistance and its impact on clinical practice. Br J Haematol 166: 471-484. doi: https://doi.org/10.1111/bjh.12896

Anderson J (2005) Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nat Rev Microbiol 3: 547–556. doi: https://doi.org/10.1038/nrmicro1179

Arendrup MC, Friberg N, Mares M, Kahlmeter G, Meletiadis J, Guinea J (2020) Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). How to interpret MICs of antifungal compounds according to the revised clinical breakpoints v. 10.0 European committee on antimicrobial susceptibility testing (EUCAST). Clin Microbiol Infect 26: 1464-1472. doi: 10.1016/j.cmi.2020.06.007

Arvanitidou M, Kanellou K, Constantinides TC, Katsouyannopoulos V (1999) The occurrence of fungi in hospital and community potable Waters. Lett Appl Microbiol 29: 81–84. doi: https://doi.org/10.1046/j.1365-2672.1999.00583.x.

Assress HA, Selvarajan R, Nyoni H. *et al.* (2021) Azole antifungal resistance in fungal isolates from wastewater treatment plant effluents. Environ Sci Pollut Res 28: 3217–3229. doi: https://doi.org/10.1007/s11356-020-10688-1

Assress HA, Nyoni H, Mamba BB, Msagati TAM (2020) Occurrence and risk assessment of azole antifungal drugs in water and wastewater. Ecotoxicol Environ Saf 187: 109868. doi: https://doi.org/10.1016/j.ecoenv.2019.109868

Assress HA, Selvarajan R, Nyoni H, Ntushelo K, Mamba BB, Msagati TAM (2019) Diversity, co-occurrence and implications of fungal communities in wastewater treatment plants. Sci Rep 9: 14056. doi: https://doi.org/10.1038/s41598-019-50624-z

Atıcı S, Soysal A, Karadeniz Cerit K, Yılmaz Ş, Aksu B, Kıyan G, Bakır M (2017) Catheter-related *Saccharomyces cerevisiae* Fungemia Following Saccharomyces boulardii Probiotic Treatment: In a child in intensive care unit and review of the literature. Med Mycol Case Rep 15: 33-35. doi: 10.1016/j.mmcr.2017.02.002

Bengtsson-Palme J, Larsson DJ (2016) Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ Int 86: 140–149. doi: 10.1016/j.envint.2015.10.015

Berman J, Krysan DJ (2020) Drug resistance and tolerance in fungi. Nat Rev Microbiol 18: 319–331. doi: https://doi.org/10.1038/s41579-019-0322-2

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases – estimate precision. J Fungi (Basel) 3: 57. doi: 10.3390/jof3040057

Bouz G, Doležal M (2021) Advances in Antifungal Drug Development: An Up-To-Date Mini Review. Pharmaceuticals 14: 1312. doi: https://doi.org/10.3390/ph14121312

Chaabane F, Graf A, Jequier L, Coste AT (2019) Review on antifungal resistance mechanisms in the emerging pathogen Candida auris. Front Microbiol 10: 2788. doi: https://doi.org/10.3389/fmicb.2019.02788

Chen ZF, Ying GG, Liu YS, Zhang QQ, Zhao JL, Liu SS, Chen J, Peng FJ, Lai HJ, Pan CG (2014) Triclosan as a surrogate for household biocides: an investigation into biocides in aquatic environments of a highly urbanized region. Water Res 58: 269e279. doi: https://doi.org/10.1016/j.watres.2014.03.072

CLSI. Performance Standards for Antifungal Susceptibility Testing of Yeasts. 1st ed. CLSI supplement M60. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.

Duggan S, Usher J (2023) *Candida glabrata*: A powerhouse of resistance. PLoS Pathog 19: e1011651. doi: https://doi.org/10.1371/journal.ppat.1011651

Enache-Angoulvant A, Hennequin C (2005) Invasive *Saccharomyces* Infection: A Comprehensive Review. Clin Infect Dis 41: 1559–1568. doi: https://doi.org/10.1086/497832

EUCAST Technical Note on fluconazole (2008) The European Committee on Antimicrobial Susceptibility Testing—Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST). doi: https://doi.org/10.1111/j.1469-0691.2007.01899.x

Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science 18:360: 739-742. doi: 10.1126/science.aap7999

Hanna N, Sun P, Sun Q, et al., (2018) Presence of antibiotic residues in various environmental compartments of Shandong province in eastern China: its potential for resistance development and ecological and human risk. Environ Int 114: 131‑142. doi: 10.1016/j.envint.2018.02.003

Kahle M, Buerge IJ, Hauser A, MüllerMD, Poiger T (2008) AzoleFungicides: Ocurence and Fate in Wastewater and surface waters. Environ Sci Technol 42: 7193-7200. doi: https://doi.org/10.1021/es8009309

Li S, Wu Q, Sun Q, Coffin S, Gui W, Zhu G (2019) Parental exposure to tebuconazole causes thyroid endocrine disruption in zebrafish and developmental toxicity in offspring. Aquat Toxicol 211: 116-123. doi: https://doi.org/10.1016/j.aquatox.2019.04.002

Liu J, Lu G, Yang H, Yan Z, Wang Y, Wang P (2016) Bioconcentration and metabolism of ketoconazole and effects on multi-biomarkers in crucian carp (Carassius auratus). Chemosphere 150: 145-151. doi: https://doi.org/10.1016/j.chemosphere.2016.02.033

Medeiros AO, Missagia BS, Brandão LR, Callisto M, Barbosa FAR, Rosa CA (2012) Water quality and diversity of yeast from tropical lakes and rivers from the Rio Doce basin in Southeastern Brazil. Braz J Microbiol 43: 1582-1594. doi: https://doi.org/10.1590/S1517-83822012000400043

Menu E, Filori Q, Dufour JC, Ranque S, L'Ollivier C (2023) A Repertoire of the Less Common Clinical Yeasts. J Fungi 9: 1099. doi: https://doi.org/10.3390/jof9111099

Monapathi ME, Bezuidenhout CC, Rhode OHJ (2017) Water quality and antifungal susceptibility of opportunistic yeast pathogens from rivers. Water Sci Technol 75: 1319–1331. doi: https://doi.org/10.2166/wst.2016.580

Monapathi ME, Horn S, Vogt T, van Wyk D, Mienie C, Ezeokoli OT, Coertze R, Rhode O, Bezuidenhout CC (2021a) Antifungal agents, yeast abundance and diversity in surface water: Potential risks to water users. Chemosphere 274: 129718. doi: https://doi.org/10.1016/j.chemosphere.2021.129718

Monapathi ME, Oguegbulu JC, Adogo L, Klink M, Okoli B, Mtunzi F, Modise JS (2021b) Pharmaceutical Pollution: Azole Antifungal Drugs and Resistance of Opportunistic Pathogenic Yeasts in Wastewater and Environmental Water. Appl Environ Soil Sci Article ID 9985398. doi: https://doi.org/10.1155/2021/9985398

Monapathi ME, Bezuidenhout CC, Rhode OHJ (2017) Water quality and antifungal susceptibility of opportunistic yeast pathogens from rivers. Water Sci Technol 75: 1319–1331. doi: https://doi.org/10.2166/wst.2016.580

Moser D, Biere K, Han B, Hoerl M, Schelling G, Choukér A, Woehrle T (2021) COVID-19 Impairs Immune Response to Candida albicans. Front Immunol 12: 640644. doi: https://doi.org/10.3389/fimmu.2021.640644

Muñoz P, Bouza E, Cuenca-Estrella M, Eiros JM, Pérez MJ, Sánchez-Somolinos M, Rincón C, Hortal J, Peláez T (2005) *Saccharomyces cerevisiae* Fungemia: An Emerging Infectious Disease. Clin Infect 40: 1625–1634. doi: https://doi.org/10.1086/429916

Munzhelele EP, Mudzielwana R, Ayinde WB, Gitari WM (2024) Pharmaceutical Contaminants in Wastewater and Receiving Water Bodies of South Africa: A Review of Sources, Pathways, Occurrence, Effects, and Geographical Distribution. Water 16: 796. doi: https://doi.org/10.3390/w16060796

Parslow BY, Thornton CR (2022) Continuing shifts in epidemiology and antifungal susceptibility highlight the need for improved disease management of invasive Candidiasis. Microorganisms 10: 1208. doi: https://doi.org/10.3390/microorganisms10061208

Perlin DS, Rautemaa-Richardson R, Alastruey-Izuierdo A (2017) The global problem of antifungal resistance: prevalence, mechanisms, and management. Lancet Infect Dis 17: e383–92. doi: http://dx.doi.org/10.1016/S1473-3099(17)30316-X

Peng X, Huang Q, Zhang K, Yu Y, Wang Z, Wang C (2012) Distribution, behaviour and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci Total Environ 426: 311-317. doi: 10.1016/j.scitotenv.2012.03.067

Pfaller MA, Diekema DJ, Sheehan DJ (2006) Interpretive breakpoints for fluconazole and Candida revisited: a blueprint for the future of antifungal susceptibility testing. Clin Microbiol Rev 19: 435-47. doi: 10.1128/CMR.19.2.435-447.2006

Pfaller MA, Rhomberg PR, Messer SA, Jones RN, Castanheira MJDM (2015) Isavuconazole, micafungin, and 8 comparator antifungal agents' susceptibility profiles for common and uncommon opportunistic fungi collected in 2013: temporal analysis of antifungal drug resistance using CLSI species-specific clinical breakpoints and proposed epidemiological cutoff values. Diagn Microbiol Infect Dis 82: 303-313. doi: https://doi.org/10.1016/j.diagmicrobio.2015.04.008

Pour AH, Salari S, Almani PGN (2018) Oropharyngeal candidiasis in HIV/AIDS patients and non-HIV subjects in the Southeast of Iran. Curr Med Mycol 4: 1-6. doi: 10.18502/cmm.4.4.379

Revie NM, Lyer KR, Robbins N, Cowen LE (2018) Antifungal Drug Resistance: Evolution, Mechanisms and Impact. Curr Opin Microbiol 45: 70–76. doi: 10.1016/j.mib.2018.02.005

Rodriguez-Tudela JL, Arendrup MC, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Denning DW, Donnelly JP, Dromer F, Fegeler W (2008) EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts: Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST)∗. CMI 14: 398-405. doi: https://doi.org/10.1111/j.1469-0691.2007.01935.x

Iancu VI, Chiriac LF, Paun I, Pirvu F, Dinu C, Kim L, Pascu LF, Niculescu M (2024) Occurrence and distribution of azole antifungal agents in eight urban Romanian waste water treatment plants. Sci Total Environ 920: 170898. doi: https://doi.org/10.1016/j.scitotenv.2024.170898

Jamiu AT, Albertyn J, Sebolai OM, Pohl CH (2021) Update on *Candida krusei*, a potential multidrug-resistant pathogen. Med Mycol 59: 14–30. doi: https://doi.org/10.1093/mmy/myaa031

Sanglard D (2016) Emerging Threats in Antifungal-Resistant Fungal Pathogens. Front. Med 3: 11. doi: 10.3389/fmed.2016.00011

Snelders E, van der Lee HAL, Kuijpers J, Rijs AJMM, Varga J, Samson RA, Mellado E, Donders ART, Melchers WJG, Verweij PE (2008) Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism. PLoS Med 5: e219. doi: 10.1371/journal.pmed.0050219

Sousa JC, Ribeiro AR, Barbosa MO, Ribeiro C, Tiritan ME, Pereira MFR, Silva AM (2019) Monitoring of the 17 EU Watch List contaminants of emerging concern in the Ave and the Sousa Rivers. Science of the Total Environment 649: 1083-1095. doi: https://doi.org/10.1016/j.scitotenv.2018.08.309

Stevenson E, Warris A, Usher J, Gow N, Gaze W, Murray A (2022) Environment Agency: Scoping review into environmental selection for antifungal resistance and testing methodology. Environment Agency, Bristol. Project number: SC200011

Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect. 2008 Apr 14: 398-405. doi: 10.1111/j.1469-0691.2007.01935.x

Toda M, Beer KD, Kuivila KM, Chiller TM, Jackson BR (2019) Trends in Agricultural Triazole Fungicide Use in the United States, 1992–2016 and Possible Implications for Antifungal-Resistant Fungi in Human Disease. Environ Health Perspect 129: CID: 055001. doi: https://doi.org/10.1289/EHP7484

Vanreppelen G, Wuyts J, Van Dijck P, Vandecruys P (2023) Sources of Antifungal Drugs. J Fungi 9: 171. doi: https://doi.org/10.3390/jof9020171

Won EJ, Choi MJ, Kim MN, Yong D, Lee WG, Uh Y, Kim TS, Byeon SA, Lee SY, Kim SH, Shin JH (2021) Fluconazole-Resistant Candida glabrata Bloodstream Isolates, South Korea, 2008-2018. Emerg Infect Dis 27: 779-788. doi: 10.3201/eid2703.203482

Yamasato K, Goto S, Ohwada K, Okuno D, Araki H, Iizuka H (1974) Yeasts from the Pacific Ocean. J Gen Appl Microbiol 20: 289-307. doi: https://doi.org/10.2323/jgam.20.289

Yang Q, Angly FE, Wang Z, Zhang H (2011) Wastewater treatment systems harbour specific and diverse yeast communities. Biochem Eng J 58– 59: 168– 176. doi: https://doi.org/10.1016/j.bej.2011.09.012

Yao J, Zhao JL, Liu YS, Yang YY, Liu WR, Ying GG (2016) Simultaneous determination of 24 personal care products in fish muscle and liver tissues using QuEChERS extraction coupled with ultra pressure liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometer analyses. Anal Bioanal Chem 408: 8177–8193. doi: https://doi.org/10.1007/s00216-016-9924-y

Chapter 5:

General discussions and conclusions

Introduction

Pathogenic yeast can have severe to fatal outcomes in immunocompromised or suppressed individuals, this prompts the need to investigate ways in which to combat fungal infections and or to prevent infections. Annually, 2.5 million deaths are due to fungal infections with approximately 6.5 million cases resulting in severe to fatal outcomes (Denning, 2024). This high number is attributed to an increase in cases of cancer, HIV/AIDS, asthma and other illnesses and treatments leading to a compromised immune system (Pour et al., 2018; Bongomin et al., 2017). Much research is dedicated to investigating antifungal interaction with these yeasts and developing novel antifungal agents, yet progress is limited.

Infections caused by *Candida* are the third leading cause of bloodstream infections and are often the culprit of nosocomial infection with an estimated 750 000 cases yearly (Bongomin et al., 2017). Cryptococcal infections account for 19% of AIDS-related deaths and are the leading cause of meningitis in individuals with HIV in low and middle-income countries (Rajasingham et al. 2022). Both yeast genera are found in the critical-risk group as outlined in the fungal priority pathogens list released by the World Health Organisation in 2022 (WHO, 2022). Major fungal pathogens like *Candida* and *Cryptococcus* species are better studied than other less common yeast species which are also capable of causing serious infections.

Numerous lesser-known pathogenic yeast species are found in the surrounding environment with which humans are in frequent contact. Due to the limited knowledge on these species, treatment of these infections is more difficult. To add to this, there is growing concern regarding the development of antifungal resistance, which leads to treatment being a greater challenge. Antifungal resistance in yeast may be intrinsic or acquired. Acquired resistance can develop due to prolonged exposure of a species to an antifungal, for example, in clinical settings, patients with fungal infections are treated with antifungals and this exposure might lead to resistance development. These resistant species may then be shed into the environment through sewage systems. It is not only clinical environments that can contribute to resistance development, other habitats in which yeast and antifungals are in close association, including polluted aquatic environments (Revie et al., 2018) and wastewater, can also contribute to this.

Knowing which pathogenic yeast species are circulating within a community may prove useful in preparation for specific fungal infections. The best way to approach this would be to surveil yeast

presence in wastewater using wastewater-based epidemiology (WBE). This is well established for bacterial and viral surveillance, but few attempts have been made to apply this to pathogenic yeasts.

This dissertation aimed to investigate if this system could be adapted to the surveillance of pathogenic yeast and what limitations would be met. We also attempted to investigate if WWTP may contribute to acquired resistance in yeast species present in the water.

Optimising a multiplex PCR system for yeast detecting in wastewater

Wastewater contains a combination of microorganisms shed by humans (intestinal tract or skin), household surfaces or runoff from the agricultural or industrial sectors. Thus, the microbial load found in wastewater influent is high and traditional methods of culture-dependent identification will be time-consuming and labour-intensive. Clinical studies often document the successful use of multiplex PCR systems to detect multiple yeast species in one sample. With this in mind, we attempted to apply this to wastewater. Multiplex PCR offers advantages such as being time-efficient and less labour-intensive than conventional PCR methods and culture-dependant techniques.

It should be noted that clinical samples often contain fewer PCR inhibitors than wastewater. This rich media presents numerous challenges in molecular analysis due to the presence of compound such as heavy metals and pharmaceuticals, that can hinder downstream applications (Yang et al., 2019; Fu et al., 2011). This makes it essential to clean samples efficiently to remove organic and inorganic substances, proteins, carbohydrates, fats, metal ions, RNAse and polyphenols that may affect the efficiency of the selected DNA polymerase (Sidstedt et al., 2020; Acharya et al., 2017; Schrader et al., 2012). There are several ways in which samples can be cleaned but the type of membrane used in the spin column during DNA extraction is important (Yang et al., 2008). After investigating different spin columns we expected the ion exchange resin (Norgen Biotek Corp Fungi/Yeast Genomic DNA Isolation Kit) to yield better results because of its higher affinity towards larger biomolecules like DNA over smaller proteins (Yang et al., 2008) but the silica membrane (OMEGA Bio-Tek E.Z.N.A. ® Water DNA Kit kit) with a higher affinity to smaller molecules (Endres et al., 2003) performed better. This might be due to the added advantage of concentrating the cells as specified by the manufacturer's protocol beforehand, something that was not done in the Norgen kit. It might also be of interest to quantify DNA extracted to compare quantitatively which kit performed best.

The second aspect that should be optimised is the polymerase. There is a need for a polymerase with high fidelity and processivity. After testing three commercially available polymerases (*Taq*, Q5® and

AccuStart II PCR ToughMix® (2x) ready mix) we concluded that Q5® High-Fidelity DNA Polymerase worked best, in that it was the only polymerase capable of amplifying multiple targets in one reaction. The better performance of the Q5® DNA polymerase might be due to its higher fidelity and processivity than *Taq* polymerase (Pezza et al., 2014; Menin and Nichols, 2013).

Another important aspect to keep in mind is the design of primers used in the PCR. Since there are multiple primer pairs the primers need to be designed in such a way to anneal at the same temperatures, not to form hairpins, or bind to each other. In this study, we did not need to design our primers since we ordered according to the primer design done by Arastehfar and colleagues (2019).

Wastewater surveillance

Wastewater is a hub of information regarding the habits and health of the community within its catchment area. Wastewater surveillance or WBE has been used in the past to monitor poliomyelitis outbreaks (Paul et al., 1939) and in recent times, to monitor COVID-19 levels (Brumfield et al., 2022; Wu et al., 2021) and detect vaccine-derived poliovirus and wild poliovirus type 1 (Wise 2022; Anis et al., 2013). This surveillance system has shown its potential, yet few attempts have been made to use this system to detect pathogenic yeast (Zulli et al., 2024; Babler et al., 2023; Barber et al., 2023; Rossi et al., 2023).

Through the combination of culture-dependant and -independent investigation, we concluded that *Candida* species were the most abundant. Furthermore, growth studies indicate that the most frequently isolated yeast species, *C. krusei*, grew the best in this complex medium and the least isolated yeast showed no growth. This indicates that wastewater in the treatment plants can support fungal growth. Wastewater treatment plants in Bloemfontein often do not work due to reasons like maintenance, loadshedding, or damage to equipment or infrastructure. This leads to water flooding the treatment plants and remaining stagnant for long periods. During these times yeast can spread to the surrounding environment. Humans and animals living close to these treatment plants walk through this water and polluted environments, exposing themselves to potentially life-threatening fungal agents. The stagnant water might also allow for enough time for yeast to proliferate.

The multiplex PCR was also able to detect the emerging pathogen of concern *C. auris*, yet this yeast was not isolated, which shows that the multiplex PCR might be more sensitive to detect cells in lower concentrations. *C. auris* was also reported by a public hospital in the area. There were also other instances where hospitals reported yeast infections that corresponds to the yeast identified in our

culture-dependant and independent data, although direct correlations could not be done. However, this suggests that WBE for pathogenic yeast may indicate which pathogens are circulating within the community.

Antifungal resistance

As previously mentioned, antifungal resistance is a global concern due to the high rate of occurrence (Berman and Krysan, 2020; Fisher et al., 2018) and the limited number of available antifungal drugs. Resistance development in yeast and the associated mechanisms are well documented (Sanglard 2016), but what is of interest is the environments in which this occurs. The clinical environment is an obvious place to look as patients with fungal infections seek treatment and the proximity of pathogenic yeast and antifungals could induce resistance development. This resistant yeast is then transferred to hospital surfaces which are washed and yeast travels through the sewer systems to treatment plants or other aquatic or terrestrial environments. However. this might not be the only environment in which this occurs. Antifungals have applications in other industries than just pharmaceuticals, they are used in agriculture as fungicides (Li et al., 2019), personal care products (Liu et al., 2016) and food preservatives. Thus, it is no surprise that they are frequent pollutants in the aquatic and terrestrial environments. Yeast and antifungal presence in aquatic habitats are frequently reported, which leads to the hypothesis that this close association might induce resistance development. We can then transfer this way of thinking to wastewater.

The concentration of fluconazole in wastewater might be linked to the season. From literature, it has been reported that fluconazole is measured at a higher concentration during the summer months than the winter months (Peng et al., 2012). The results from our study also correspond to the literature. This may be due to summer months being warmer and more humid, creating more favorable conditions for the growth of yeast leading to a higher infection rate and thus higher concentrations of fluconazole in wastewater.

The month with the second-highest concentration of fluconazole also yielded the most resistant yeast strain isolated over the 12 months. This could indicate that wastewater is inducing antifungal resistance development, but an alternative explanation may be that the high fluconazole concentration is already selecting for more resistant strains to proliferate. This is supported by the abundant occurrence of *C. krusei* in March 2024, which is intrinsically resistant to fluconazole. *C. tropicalis* and *C. albicans* isolates were susceptible to fluconazole and *C. glabrata* was susceptible dose-dependent with one resistant strain. Thus, we can conclude that WWTPs contain and support the

growth of pathogenic yeast species that range from susceptible, susceptible dose-dependent to resistant. This alone can pose a serious health risk to individuals and animals who come into contact with this wastewater.

Limitations and future considerations

Throughout this study, we identified certain limitations to the experimental work presented. Some of these limitations were out of our control while others were experimental limitations that future researchers should strive to improve on.

With regards to the multiplex PCR, it is still unclear if the primers were able to identify two yeast species, *Trichosporon lactis* and *Candida dubliniensis*, as these were not detected by this technique in any of the wastewater samples. In future studies, it would be worthwhile to expand on the validation of this PCR, which was initially designed for clinical samples. Potential ways to do this include spiking filtered wastewater samples with pure cultures of the intended yeast targets to see if the multiplex PCR would amplify the genetic material of all the yeast, and performing a gradient PCR to ensure that the optimal annealing temperature is used.

In the growth studies, it should be considered that the physiochemical parameters were not altered during the experiment, this means that the samples still contained residual antifungal agents and other chemicals. This might be why yeasts such as *C. krusei*, grew better than others and future studies might choose to remove the antifungals, yet those samples would not mimic the actual environment in wastewater treatment plants since it will always contain a variety of pharmaceuticals and chemicals.

The hospital data obtained from several public hospitals was also difficult to interpret, because this was often incomplete. We could thus not make any conclusions linking hospital reports on fungal infections to the data obtained from the culture-independent and -dependent data. This also highlighted the need for hospitals in the Bloemfontein area to update methods of isolate identification. Culture mycology was used in each case but is notoriously unreliable. Switching to molecular-based identification could bypass culture mycology limitations and yield quicker more reliable results, yet it is not without its challenges like proper training and expensive equipment.

In conclusion, it does seem possible to use wastewater surveillance to monitor pathogenic yeast in the community, although there are many confounding issues that need to be taken into consideration and more research is needed to optimise and validate this system before it can be implemented. Importantly, in the current context in South Africa and even in developed countries, such as the United

Kingdom and United States of America, where sewage spills are reported, wastewater can act as a source of pathogenic yeast (including those exhibiting drug resistance) which pose a health risk to immunocompromised or suppressed individuals in the surrounding environment.

References

Acharya KR, Dhand NK, Whittington RJ, Plain KM (2017) PCR Inhibition of a Quantitative PCR for Detection of Mycobacterium avium Subspecies Paratuberculosis DNA in Feces: Diagnostic Implications and Potential Solutions. Front Microbiol 8: 115. doi: 10.3389/fmicb.2017.00115

Anis E, Kopel E, Singer SR, Kaliner E, Moerman L, Moran-Gilad J, Sofer D, Manor Y, Shulman LM, Medelson E, Gdalevich M, Lev B, Gamzu R, Grotto I (2013) Insidious reintroduction of wild poliovirus into Israel. Eurosurveillance 18: 20586. doi: https://doi.org/10.2807/1560-7917.ES2013.18.38.20586

Arastehfar A, Carvalho A, Nguyen MH, Hedayati MT, Netea MG, Perlin DS, Hoenigl M (2020) COVID-19-Associated Candidiasis (CAC): An Underestimated Complication in the Absence of Immunological Predispositions? J Fungi 6: 211. doi: https://doi.org/10.3390/jof6040211

Berman J, Krysan DJ (2020) Drug resistance and tolerance in fungi. Nat Rev Microbiol 18: 319–331. doi: https://doi.org/10.1038/s41579-019-0322-2

Brumfield KD, Leddy M, Usmani M, Cotruvo JA, Tien CT, Dorsey S, Graubics K, Fanelli B, Zhou I, Registe N, Dadlani M (2022) Microbiome analysis for wastewater surveillance during COVID-19. mBio 13: e00591-22. doi: https://doi.org/10.1128/mbio.00591-22

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases – estimate precision. J Fungi 3: 57. doi: https://doi.org/10.3390/jof3040057

Denning DW (2024) Global incidence and mortality of severe fungal disease. Lancet infect Dis 24: e428-38. doi: https://doi.org/10.1016/ S1473-3099(23)00692-8

Endres HN, Johnson JAC, Ross CA, Welp JK, Etzel MR (2003). Evaluation of an ion-exchange membrane for the purification of plasmid DNA. Biotechnol Appl Biochem 37: 259-266. doi: 10.1042/ba20030025

Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ (2018) Worldwide emergence of resistance to antifungal drugs challenges human health and food security. Science 18:360: 739-742. doi: 10.1126/science.aap7999

Fu F, Wang Q (2011) Removal of heavy metal ions from wastewater: A review. JEM 92: 407-418. doi: https://doi.org/10.1038/s41545-021-00127-0

Kainz K, Bauer M A, Madeo F, Carmona-Gutierrez D (2020) Fungal infections in humans: the silent crisis. Microbial Cell 7: 143-145. doi: https://doi.org/10.15698/mic2020.06.718

Li S, Wu Q, Sun Q, Coffin S, Gui W, Zhu G (2019) Parental exposure to tebuconazole causes thyroid endocrine disruption in zebrafish and developmental toxicity in offspring. Aquat Toxicol 211: 116-123. doi: https://doi.org/10.1016/j.aquatox.2019.04.002

Liu J, Lu G, Yang H, Yan Z, Wang Y, Wang P (2016) Bioconcentration and metabolism of ketoconazole and effects on multi-biomarkers in crucian carp (*Carassius auratus*). Chemosphere 150: 145-151. doi: https://doi.org/10.1016/j.chemosphere.2016.02.033

Menin JF and Nichols NM (2013) Multiplex PCR using Q5® High-Fidelity DNA Polymerase. N Engl Biolabs Appl. Note.

Paul JR, Trask JD, Culotta CS (1939) Poliomyelitic virus in sewage. Science 90: 258-259

Peng X, Huang Q, Zhang K, Yu Y, Wang Z, Wang C (2012) Distribution, behaviour and fate of azole antifungals during mechanical, biological, and chemical treatments in sewage treatment plants in China. Sci Total Environ 426: 311-317. doi: 10.1016/j.scitotenv.2012.03.067

Pezza JA, Kucera R, Sun L (2014) Polymerase fidelity: what is it, and what does it mean for your PCR. *New England Biolabs*

Pour AH, Salari S, Almani PGN (2018) Oropharyngeal candidiasis in HIV/AIDS patients and non-HIV subjects in the Southeast of Iran. Current Medical Mycology 4: 1-6. doi: https://doi.org/10.18502/cmm.4.4.379

Rajasingham R, Govender NP, Jordan A, Loyse A, Shroufi A, Denning DW, Meya DB, Chiller TM, Boulware DR (2022) The global burden of HIV-associated cryptococcal infection in adults in 2020: a modelling analysis. The Lancet Infect Dis 22: 1748-1755. doi: https:/doi.org/10.1016/S1473-3099(22)00499-6

Revie NM, Lyer KR, Robbins N, Cowen LE (2018) Antifungal Drug Resistance: Evolution, Mechanisms and Impact. Curr Opin Microbiol 45: 70–76. doi: 10.1016/j.mib.2018.02.005

Sanglard D (2016) Emerging Threats in Antifungal-Resistant Fungal Pathogens. Front Med 3: 11. doi: 10.3389/fmed.2016.00011

Schrader C, Schielke A, Ellerbroek L, Johne R (2012) PCR inhibitors - occurrence, properties and removal. J Appl Microbiol 113: 1014–1026. doi: doi:10.1111/j.1365-2672.2012.05384.x

Sidstedt M, Rådström P, Hedman J (2020) PCR inhibition in qPCR, dPCR and MPS-mechanisms and solutions. Anal Bioanal Chem 412: 2009-2023. doi: 10.1007/s00216-020-02490-2

Wise J (2022) Poliovirus is detected in sewage from north and east London. BMJ 377. doi: https://doi.org/10.1136/bmj.o1546

Wu F, Xiao A, Zhang J (2021) Wastewater surveillance of SARS-CoV-2 across 40 U.S. states from February to June 2020. Water Res 202: 117400. doi: https://doi.org/10.1016/j.watres.2021.117400

WHO fungal priority pathogens list to guide research, development and public health action. Geneva: World Health Organization; 2022. Licence: CC BY-NC-SA 3.0 IGO

Yang J, Hou B, Wang J, Tian B, Bi J, Wang N, Li X, Huang X (2019) Nanomaterials for the removal of heavy metals from wastewater. Nanomaterials 9: 424. doi: https://doi.org/10.3390/nano9030424

Yang Y, Hebron HR, Hang J (2008) High performance DNA purification using a novel ion exchange matrix. J Biomol Tech 19: 205-210

Zulli A, Chan EMG, Shelden B, Duong D, Xu X-RS, White BJ, Wolfe MK, Boehm AB (2024) Prospective study of *Candida auris* nucleic acids in wastewater solids in 190 wastewater treatment plants in the United States suggests widespread occurrence. mBio 15: e0090824. doi: 10.1128/mbio.00908-24

Summary

Pathogenic yeast poses a serious health risk to immunocompromised or suppressed individuals with outcomes ranging from superficial, severe to fatal. Fungal infections have frequently been overlooked with more attention being paid to viral and bacterial infections. Due to this neglect, there is a lack of knowledge about these microorganisms and how to combat their outcomes. Questions on metabolism, antifungal resistance and natural reservoirs need to be answered. Antimicrobial resistance is a growing concern and is not limited to the clinical environment but has also been documented in aquatic environments such as freshwater or wastewater. Wastewater is a collection point for numerous pathogens and pharmaceuticals shed by the human intestinal tract, agricultural and industrial run-off. This close association might lead to the development of antimicrobial resistance as seen in bacteria, but the same effect is only speculated in yeast. Considering the potentially severe outcomes associated with pathogenic yeast and their occurrence in wastewater, it is necessary to know which yeasts are circulating in the community and if they are potentially resistant to common antifungals. This information may be important for epidemiology and to ensure the efficient treatment of patients.

This study aimed to investigate which yeast are circulating within a community using wastewater-based epidemiology/ wastewater surveillance, a system not yet optimised for pathogenic yeast (or at least multiple strains). This was done by isolating and identifying yeast with Sanger sequencing for culture-dependent studies. For culture-independent analysis, a clinical multiplex PCR was adapted to detect yeast in the wastewater. This data was then compared to hospital reports on fungal infections during the same time. Antifungal resistance of the yeast isolated from wastewater was done using microbroth dilutions with fluconazole according to the CLSI protocol. Fluconazole concentrations were also determined using LC/MS and finally, growth studies were performed to determine if yeast can proliferate in wastewater.

These results showed the diversity of pathogenic yeast that can occur in wastewater. One-hundred-and-sixty-six yeast isolates were obtained and identified, with *Candida krusei* as the most frequently isolated. The multiplex PCR revealed *Candida glabrata* to be the most prevalent and was also able to detect *Candida auris* which might indicate that the PCR has an increased sensitivity to detect cells in low concentration. Fluconazole concentrations were generally higher during the summer months in South Africa than the winter months which may be due to the more favorable conditions for yeast growth during summer, leading to higher levels of fluconazole use in the community. The microbroth

dilutions highlighted the intrinsic resistance displayed by *C. krusei* and point to wastewater possibly selecting for resistant strains. In conclusion, wastewater surveillance to monitor pathogenic yeast shows potential but more studies are needed before this system can applied in practice.

Supplementary data

Table of Contents

Chapter 2:
Optimisation of experimental procedures on wastewater samples

Multiplex Polymerase chain reaction (PCR)

Table S3 List of yeast isolates with their corresponding Primer name and sequences

Yeast isolates with SANBI reference number	**Primer Name**	**Primer sequence**
First set (Mx1)		
Candida albicans SC 5314	**Falb**	AGATTATTGCCATGCCCTGAG
	Ralb	CCATGTCGAACGTAGCGTAT*
Candida auris UOFS Strain 293	**Faur**	GAACGCACATTGCGCCTTGG
	Raur	TCCAAAGGACTTGCCTGCT
Candida dubliniensis UOFS 2778, NRRL-1-17841	**Fdub**	GTCGGACATATACCTCCAACTC
	Rdub	CCATGTCGAACGTAGCGTAT*
Candida glabrata UOFS 0870, CSIR 0168	**Fgla**	ACCGTGCTTGCCTCTACA
	Rgla	GACATCTGAGCCTCGTCTGA
Candida parapsilosis UOFS 0569, TT17	**Fpara**	TACACCAAGCGACTCAGC
	Rpara	ACCAGCTGCTTTGACTTG
Pichia kudriavzevii/ Candida krusei UOFS-1-2457, MOH 085	**Fkru**	GGCGTTGTCCATCCAATG
	Rkru	CAGGAGAATTGCTGTTCCC
Candida tropicalis UOFS 0534, OC3	**Ftro**	AGAACAAGAAAACAGTGAAGCAA
	Rtro	CCATGTCGAACGTAGCGTAT*
Second set (Mx2a and Mx2b)		
Debaromyces hansenii UOFS 1991, CBS 1795	**Fam-F**	GGATCTCTTGGTTCTCGCA*
	Fam-R	GCGAGGAACCCAACCAAGA
Meyerozyma guilliermondi UOFS0209, CBS 2030, NRRLY-02075	**Guil-F**	GGATCTCTTGGTTCTCGCA*
	Guil-R	CCAGAAATATCCCGCCACA
Kluyveromyces marxianus UOFS 1191, IGC 2671	**Kef-F**	GGATCTCTTGGTTCTCGCA*
	Kef-R	ACTTTCAAGTTAACCCGAGAC

Yarrowia lipolytica UOFS 1139, CBS 0599	**Lipo-F**	ACCGAGAGCGACGAGTA
	Lipo-R	CTTTCTACCCAGAGCCACAA
Clavisopra lusitaniae UOFS 0655, TT11	**Lusi-F**	GGATCTCTTGGTTCTCGCA*
	Lusi-R	CCGACTCAGACCACGAAAC
Pichia norvegensis UOFS 1561, CSIR-1-0923	**Norve-F**	GGGTTTGGAACCAATCTCAGA
	Norve-R	GCAAAATCGGTGTTTTCGCTG
Diutina rugosa UOFS 1143, CSIR-1-0788	**Rugo-F**	GGATCTCTTGGTTCTCGCA*
	Rugo-R	ACGGCCTTTTCACGAGAAGG
Third set (Mx3)		
Cryptococcus deneoformans*	**Deneo-F**	CCATCCTGTTGGCGAAGA*
	Deneo-R	GGTGCTGTATGAAGGCTATGG**
Cryptococcus neoformans UOFS 3240, CAB 843	**Neo-F**	CCATCCTGTTGGCGAAGA*
	Neo-R	GGTGCTGTATGAAGGCTATGG**
Cryptococcus gattii UOFS 2933, CAB 851	**Gattii-F**	CAGGAGTGGATTCAGCGT
	Gattii-R	GGTGCTGTATGAAGGCTATGG**
Geotrichum candidum*	**Gcandi-F**	AGATTGTATCTTGAGAGCGGATTA
	Gcandi-R	GCCGAAACACAGTTGAACAA
Rhodotorula mucliginosa UOFS 3016, O13G	**Rhodo-F**	GCCTAGCTCGTTCGTAATGC
	Rhodo-R	TTAACCCAACCCGGCTCT
Trichosporon asahii UOFS 2273, SA519/SB5	**Tasahii-F**	GAAGGATCATTAGTGATTGCCTT
	Tasahii-R	TACCTGATTTCAGGCCAGAG
Trichosporon lactis*	**Tlactis-F**	GAAGGATCATTAGTGATTGCCTT
	Tlactis-R	TACCTGATTTCAGGCCAGAG

*Not all the cultures for multiplex three as stated in the article by Arastehfar et al. (2019) were available. Cultures not obtained include *Trichosporon lactis, Geotrichum candidum* and *Cryptococcus deneoformans.*

Primer map results

Forward primers able to bind to the target sequence are indicated in purple and reverse sequences in yellow. Asterisks (*) indicate where there is a base difference between the designed primers and the target sequence.

Results for linear 606 residue sequence "*C._albicans*" starting "AGATTATTGC"

>>>Falb>>> 1 to 21

```
   1 AGATTATTGCCATGCCCTGAGGATGAGTTTAGTTTTTTAAAAATTAAAAAATGTCCAAAA
   1        10        20        30        40        50
   1 TCTAATAACGGTACGGGACTCCTACTCAAATCAAAAAATTTTTAATTTTTTACAGGTTTT
  61 CTGGTTGTGCTGTATAGGAGGGGTAAGAATTTGCCATTCTGCCCCTTTGGGTGGGTCAGT
  61        70        80        90       100       110
  61 GACCAACACGACATATCCTCCCCATTCTTAAACGGTAAGACGGGGAAACCCACCCAGTCA
 121 CAAAAAAAGAGGTATCACTCTGGTTCAAACGGGAAACAACAGAAAATGGGATAAAAATAA
 121        130       140       150       160       170
 121 GTTTTTTTCTCCATAGTGAGACCAAGTTTGCCCTTTGTTGTCTTTTACCCTATTTTTATT
 181 TCTCCAGACCAAACTTAGTAGTAACAGCCATTTTAGTTGTACTGGTATACCCTACACAAG
 181        190       200       210       220       230
 181 AGAGGTCTGGTTTGAATCATCATTGTCGGTAAAATCAACATGACCATATGGGATGTGTTC
 241 TTGTCCATTTTGTATGGGGAAGGGGAATTTAGACAAAATTTTTTTTTGAATTTCGCTAAG
 241        250       260       270       280       290
 241 AACAGGTAAAACATACCCCTTCCCCTTAAATCTGTTTTAAAAAAAAACTTAAAGCGATTC
 301 TGTCAAGACCCGCAAAAGTCACCTTTTTTCGTTTTCAACTATGGCAGAGGCTCACCTTTT
 301        310       320       330       340       350
 301 ACAGTTCTGGGCGTTTTCAGTGGAAAAAAGCAAAAGTTGATACCGTCTCCGAGTGGAAAA
 361 GTCTGCTGCACAGCCAAATTGATTTTGTAGGTGCGCACTGGAAAAATAGTTTGTTAGTGG
 361        370       380       390       400       410
 361 CAGACGACGTGTCGGTTTAACTAAAACATCCACGCGTGACCTTTTTATCAAACAATCACC
 421 ACACGTTTTTGCAGTGTGAAACTGCGCTCGGAGGTACTATATGCGAAAGCAGAAAAGACA
 421        430       440       450       460       470
 421 TGTGCAAAAACGTCACACTTTGACGCGAGCCTCCATGATATACGCTTTCGTCTTTTCTGT
 481 ATTGCAAGAATACAGAGAGTTCTTCTCTGGGCTATTGCAATGTGTTTAAGGCCAAGTCGA
 481        490       500       510       520       530
 481 TAACGTTCTTATGTCTCTCAAGAAGAGACCCGATAACGTTACACAAATTCCGGTTCAGCT
 541 CGAGTGGGGAGAGTCTGGAAGTGATATACACATCACGACCTACTTTATACGCTACGTTCG
 541        550       560       570       580       590
```

541 GCTCACCCCTCTCAGACCTTCACTATATGTGTAGTGCTGGATGAAATATGCGATGCAAGC

601 GCATGG

601 *

601 CGTACC

Results for linear 331 residue sequence "*C._auris*" starting "GAACGCACAT"

>>>Faur>>> 1 to 20

1 GAACGCACATTGCGCCTTGGGGTATTCCCCAAGGCATGCCTGTTTGAGCGTGATGTCTTC

1 10 20 30 40 50

1 CTTGCGTGTAACGCGGAACCCCATAAGGGGTTCCGTACGGACAAACTCGCACTACAGAAG

61 TCACCAATCTTCGCGGTGGCGTTGCATTCACAAAATTACAGCTTGCACGAAAAAAATCTA

61 70 80 90 100 110

61 AGTGGTTAGAAGCGCCACCGCAACGTAAGTGTTTTAATGTCGAACGTGCTTTTTTTAGAT

121 CGCTTTTTTTTTCGTTTTGTTGTCGCCTCAAATCAGGTAGGACTACCCGCTGAACTTAAG

121 130 140 150 160 170

121 GCGAAAAAAAAAGCAAAACAACAGCGGAGTTTAGTCCATCCTGATGGGCGACTTGAATTC

181 CATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTCAGTAACGGCGAGTGAAG

181 190 200 210 220 230

181 GTATAGTTATTCGCCTCCTTTTCTTTGGTTGTCCCTAACGGAGTCATTGCCGCTCACTTC

241 CGGCAAGAGCTCAACTTTGGAATCGCTCCGGCGAGTTGTAGTCTGGAGGTGGCCACCACG

241 250 260 270 280 290

241 GCCGTTCTCGAGTTGAAACCTTAGCGAGGCCGCTCAACATCAGACCTCCACCGGTGGTGC

<<<Raur<<< 313 to 331

301 AGGTGTTCTAGCAGCAGGCAAGTCCTTTGGA

301 310 320 330

301 TCCACAAGATCGTCGTCCGTTCAGGAAACCT

Results for linear 718 residue sequence "*C._dubliniensis*" starting "GTCGGACATA"

>>>Fdub>>> 1 to 22

1 GTCGGACATATACCTCCAACTCCGGTCACGTGACCAGGACTCAAAAGAAAAAAATATGAA

1 10 20 30 40 50

1 CAGCCTGTATATGGAGGTTGAGGCCAGTGCACTGGTCCTGAGTTTTCTTTTTTTATACTT

61 ATTTTCAAAATTTCAAAATCATCAAAAACAAAGTATACAGTGATTATTGCCATGCCTTGG

61 70 80 90 100 110

61 TAAAAGTTTTAAAGTTTTAGTAGTTTTTGTTTCATATGTCACTAATAACGGTACGGAACC

121 TGATGTATTTTATTTTTTTAAATTTTTTAAATTTGCAAAAATGAATGCGCTGCCCAAAGG

121 130 140 150 160 170

121 ACTACATAAAATAAAAAAATTTAAAAAATTTAAACGTTTTTACTTACGCGACGGGTTTCC

181 GGGTGGTAGTTTTGCAAATTAAGTTGATCTGCCCTTTGGGTGGGTCAGTCAAAAAAAGAG

181 190 200 210 220 230

181 CCCACCATCAAAACGTTTAATTCAACTAGACGGGAAACCCACCCAGTCAGTTTTTTTCTC

241 CTACCACCCTGGTTCAATCGGGAACTAACCTAATTATGGCTTAAAAATAATATACAAACT

241 250 260 270 280 290

241 GATGGTGGGACCAAGTTAGCCCTTGATTGGATTAATACCGAATTTTTATTATATGTTTGA

301 GTGTTTGGGTGCATCAGCCATTTTTGTTGTAATGATATACCCTCTACAAGTTACCATTTT

301 310 320 330 340 350

301 CACAAACCCACGTAGTCGGTAAAAACAACATTACTATATGGGAGATGTTCAATGGTAAAA

361 TGTATGGGAAAGGGGAAAATTCTGAAATTTTTTTTTTTGATTTTTCGCTAAGTGTCAAGA

361 370 380 390 400 410

361 ACATACCCTTTCCCCTTTTAAGACTTTAAAAAAAAAAACTAAAAAGCGATTCACAGTTCT

421 CCCCTAAAAGTCACCTTTTTTCGTTTTCAACTATGGGAGAGACGCACCTTTTATTATGCG

421 430 440 450 460 470

421 GGGGATTTTCAGTGGAAAAAAGCAAAAGTTGATACCCTCTCTGCGTGGAAAATAATACGC

481 CAAAGCCAATCCATCTTTGTGGCCACGCACTGGAAAAATAGTTTATTACGAGACATGTTC

481 490 500 510 520 530

481 GTTTCGGTTAGGTAGAAACACCGGTGCGTGACCTTTTTATCAAATAATGCTCTGTACAAG

541 TCGCAATGTGAAATGTCGCTCGGAGGTACTATATGCGAAAGCAGAAAAGACAATTGCAAG

541 550 560 570 580 590

541 AGCGTTACACTTTACAGCGAGCCTCCATGATATACGCTTTCGTCTTTTCTGTTAACGTTC

601 AATACAGAGAGTTCTTCTCTGGGCTACTGCAATCTGTTTAAGGCCAAGTCGACGAGTGGG

601 610 620 630 640 650

601 TTATGTCTCTCAAGAAGAGACCCGATGACGTTAGACAAATTCCGGTTCAGCTGCTCACCC

<<<Ralb<<< 699 to 718

<<<Rdub<<< 699 to 718

<<<Rtro<<< 699 to 718

661 GAGAGTCTGGAAGTGATATATACATCACGACCTACTTTATACGCTACGTTCGACATGG

661 670 680 690 700 710

661 CTCTCAGACCTTCACTATATATGTAGTGCTGGATGAAATATGCGATGCAAGCTGTACC

Results for linear 212 residue sequence "*C._glabrata*" starting "ACCGTGCTTG"

>>>Fgla>>> 1 to 18

1 ACCGTGCTTGCCTCTACATCTGCTGATCACACCGTTAAGCTATGGGACTTGAACAACGCT

1 10 20 30 40 50

1 TGGCACGAACGGAGATGTAGACGACTAGTGTGGCAATTCGATACCCTGAACTTGTTGCGA

61 ACTGCTGCACGTTCCATGGCCAACATCCACAGCAACAAGAACGTCTCCTCGTCTCAGTGG

61 70 80 90 100 110

61 TGACGACGTGCAAGGTACCGGTTGTAGGTGTCGTTGTTCTTGCAGAGGAGCAGAGTCACC

121 CACATGCAGAACGCTTCAATCTTGCTGACTGCAGGTTACGATTCCCGTGCTGCGTTGACC

121 130 140 150 160 170

121 GTGTACGTCTTGCGAAGTTAGAACGACTGACGTCCAATGCTAAGGGCACGACGCAACTGG

<<<Rgla<<< 193 to 212

181 GATGTTAGAATATCAGACGAGGCTCAGATGTC

181 190 200 210

181 CTACAATCTTATAGTCTGCTCCGAGTCTACAG

Results for linear 490 residue sequence "*C._parapsilosis*" starting "TACACCAAGC"

>>>Fpara>>> 1 to 18

1 TACACCAAGCGACTCAGCCCCCATAGTGCTTATAAGCAATTGAGCCATCCCTCGTGCAGA

1 10 20 30 40 50

1 ATGTGGTTCGCTGAGTCGGGGGTATCACGAATATTCGTTAACTCGGTAGGGAGCACGTCT

61 AACGATAATCATGCTTTTCTGCATAGCACTTTATCGCACCTATGACCATTCACCAAGCTA

61 70 80 90 100 110

61 TTGCTATTAGTACGAAAAGACGTATCGTGAAATAGCGTGGATACTGGTAAGTGGTTCGAT

121 TTGGGTAGCGGACCATTGCGCAATTAATGGCCATGATAAGAAACACATCTGTCTGCAAAA

121 130 140 150 160 170

121 AACCCATCGCCTGGTAACGCGTTAATTACCGGTACTATTCTTTGTGTAGACAGACGTTTT

181 CAGGTTTCTTCTAAATCAAACTAATAATACATTCTGTACATAATGATACAAATTTCTAAA

181 190 200 210 220 230

181 GTCCAAAGAAGATTTAGTTTGATTATTATGTAAGACATGTATTACTATGTTTAAAGATTT

241 CGCAATGTTTTACATCATCTAAAAAGCAAAACGTTCCAACTTGAGCAATCCGCCCAAAGT

241 250 260 270 280 290

241 GCGTTACAAAATGTAGTAGATTTTTCGTTTTGCAAGGTTGAACTCGTTAGGCGGGTTTCA

301 AACCTTATCTCTCTTAACATCTGGACCCAAATCAAAGCCCAAAATTGCGTGCAATGCAGC

301 310 320 330 340 350

301 TTGGAATAGAGAGAATTGTAGACCTGGGTTTAGTTTCGGGTTTTAACGCACGTTACGTCG

361 TTCAAAGTAATTCTTGATTGATTGTGGAGTTCCTGGATATTCCAAGTTTGAAGCTCTCAC

361 370 380 390 400 410

361 AAGTTTCATTAAGAACTAACTAACACCTCAAGGACCTATAAGGTTCAAACTTCGAGAGTG

<<<Rpara<<< 473 to 490

421 ATTATGTTGACATCCATCAACTGGAGGCTGTCCATTGAAACGATTGATAATCCAAGTCAA

421 430 440 450 460 470

421 TAATACAACTGTAGGTAGTTGACCTCCGACAGGTAACTTTGCTAACTATTAGGTTCAGTT

481 AGCAGCTGGT

481

481 TCGTCGACCA

Results for linear 1159 residue sequence "*P._kudriavzevii*" starting "GGCGTTGTCC"

>>>FKru>>> 1 to 18

1 GGCGTTGTCCATCCAATGAGAAGGGGGAACAGGATGATCAAATCCTTGAGACCCGGAGGA

1 10 20 30 40 50

1 CCGCAACAGGTAGGTTACTCTTCCCCCTTGTCCTACTAGTTTAGGAACTCTGGGCCTCCT

61 GCTACAAACTACTCAACGACATCGACACCAACTTCGTCGACACCGTCAGCTACATCACCT

61 70 80 90 100 110

61 CGATGTTTGATGAGTTGCTGTAGCTGTGGTTGAAGCAGCTGTGGCAGTCGATGTAGTGGA

121 ACACATTCTGCGACACACCTATCTGCTTCCACATCAAACATCCATCAGAACAGTGCAAGT

121 130 140 150 160 170

121 TGTGTAAGACGCTGTGTGGATAGACGAAGGTGTAGTTTGTAGGTAGTCTTGTCACGTTCA

181 GCCAGACATGCGGCAAACTTGATCTGGTCGCCCTCAAACTCATTGACCCCAAGGGACGAG

181 190 200 210 220 230

181 CGGTCTGTACGCCGTTTGAACTAGACCAGCGGGAGTTTGAGTAACTGGGGTTCCCTGCTC

241 TTTGCGGATTTCTCCTCAACTTCAACCATTAGTGATCGCTCCAAGGCTGCATTAAAGGCT

241 250 260 270 280 290

241 AAACGCCTAAAGAGGAGTTGAAGTTGGTAATCACTAGCGAGGTTCCGACGTAATTTCCGA

301 GCAGCCAAAGTGAAGCCCCCAAGACGCCCATACAAACCTAAACCAGAGCAAACAAGATCC

301 310 320 330 340 350

301 CGTCGGTTTCACTTCGGGGGTTCTGCGGGTATGTTTGGATTTGGTCTCGTTTGTTCTAGG

361 TTTGAAAATAAATCACCACCAACAACAAATGCATCCAGATCAAACAGTCCTGCATCACAC

361 370 380 390 400 410

361 AAACTTTTATTTAGTGGTGGTTGTTGTTTACGTAGGTCTAGTTTGTCAGGACGTAGTGTG

421 TCGGCACCTCCGGAATTGGACCCTGAATTTGATCATAATTACAGTGGAGGATTACCACCA

421 430 440 450 460 470

421 AGCCGTGGAGGCCTTAACCTGGGACTTAAACTAGTATTAATGTCACCTCCTAATGGTGGT

481 TCGAGACGGGGGAAACAACCACAGATTCGAGGCAAAGATTCACAACAACCTCCATTGGAA

481 490 500 510 520 530

481 AGCTCTGCCCCCTTTGTTGGTGTCTAAGCTCCGTTTCTAAGTGTTGTTGGAGGTAACCTT

541 GCTGTTGAACCTTCTAAGGATGAACTGAACGATGATGATGCACTTTTAAAGAAAATCAAG

541 550 560 570 580 590

541 CGACAACTTGGAAGATTCCTACTTGACTTGCTACTACTACGTGAAAATTTCTTTTAGTTC

601 GGTGTTGATATTAGATTCACAAGGAGTATCTTGATGGAATCCAATAGAGACCAAGATAAG

601 610 620 630 640 650

601 CCACAACTATAATCTAAGTGTTCCTCATAGAACTACCTTAGGTTATCTCTGGTTCTATTC

661 GTGTATTGGTCCGACATTGCTGGTTTAGAACAGGCAAAGGAGTCGTTGATGGAGACTGTT

661 670 680 690 700 710

661 CACATAACCAGGCTGTAACGACCAAATCTTGTCCGTTTCCTCAGCAACTACCTCTGACAA

721 GTTTATCCATTTTTACGGCCAGACCTATTTAGAGGGTTACGAGAACCAGTTACTGGAATG

721 730 740 750 760 770

721 CAAATAGGTAAAAATGCCGGTCTGGATAAATCTCCCAATGCTCTTGGTCAATGACCTTAC

781 CTACTATTTGGTCCTCCTGGTACCGGTAAGACCATGCTTGCACGCGCAGCGGCAACCGAA

781 790 800 810 820 830

781 GATGATAAACCAGGAGGACCATGGCCATTCTGGTACGAACGTGCGCGTCGCCGTTGGCTT

841 TCCAACTCCACTTTCTTTAGTATCCAATCATCATCGTTGGCAAGTAAATGGTATGGTGAG

841 850 860 870 880 890

841 AGGTTGAGGTGAAAGAAATCATAGGTTAGTAGTAGCAACCGTTCATTTACCATACCACTC

901 TCTGAACAACTAGTACGTGCCTTATTTGAAGTCGCCAAAGCCAAGGCGCCATCAATTATT

901 910 920 930 940 950

901 AGACTTGTTGATCATGCACGGAATAAACTTCAGCGGTTTCGGTTCCGCGGTAGTTAATAA

961 TTTGTTGATGAAATCGATTCCATATTAGGCCAACGTTCAGGTGATGGAGAGGACAATGCT

961 970 980 990 1000 1010

961 AAACAACTACTTTAGCTAAGGTATAATCCGGTTGCAAGTCCACTACCTCTCCTGTTACGA

1021 GCATCAAGAGTGAAGAATGAGTTTTTGGTTCAATGGTCCGACTTATCTAAGGCAGCTGCT

1021 1030 1040 1050 1060 1070

1021 CGTAGTTCTCACTTCTTACTCAAAAACCAAGTTACCAGGCTGAATAGATTCCGTCGACGA

1081 GGTCGAGTTGATGATGAAGGTGACAATGGTAATGGTGATAGTAATGGAGATGGCAATGTG

1081 1090 1100 1110 1120 1130

1081 CCAGCTCAACTACTACTTCCACTGTTACCATTACCACTATCATTACCTCTACCGTTACAC

<<<Rkru<<< 1141 to 1159

1141 GGGAACAGCAATTCTCCTG

1141 1150

1141 CCCTTGTCGTTAAGAGGAC

Results for linear 126 residue sequence "*C._tropicalis*" starting "AGAACAAGAA"

>>>Ftro>>> 1 to 23

1 AGAACAAGAAAACAGTGAAGCAATTCATTGGAATATTGAGCTTGTTTAAGGCCAAGTCGA

1 10 20 30 40 50

1 TCTTGTTCTTTTGTCACTTCGTTAAGTAACCTTATAACTCGAACAAATTCCGGTTCAGCT

61 CGAGTGGGGAGAGTCTGGAAGGGATATACACATCCTGACCTACTTTATACGCTACGTTCG

61 70 80 90 100 110

61 GCTCACCCCTCTCAGACCTTCCCTATATGTGTAGGACTGGATGAAATATGCGATGCAAGC

121 GCATGG

121 *

121 CGTACC

Results for linear 818 residue sequence "D_hansenii" starting "GGATCTCTTG"

>>>Guil-F>>> 1 to 19

>>>Kef-F>>> 1 to 19

>>>Lusi-F>>> 1 to 19

>>>Fam-F>>> 1 to 19

>>>Rugo-F>>> 1 to 19

1 GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATT

1 10 20 30 40 50

1 CCTAGAGAACCAAGAGCGTAGCTACTTCTTGCGTCGCTTTACGCTATTCATTATACTTAA

61 GCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGAG

61 70 80 90 100 110

61 CGTCTAAAAGCACTTAGTAGCTTAGAAACTTGCGTGTAACGCGGGAGACCATAAGGTCTC

121 GGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCTTCGGGTTTGGTATTGAGTGATAC

121 130 140 150 160 170

121 CCGTACGGACAAACTCGCAGTAAAGAGAGAGTTTGGAAGCCCAAACCATAACTCACTATG

181 TCTTAGTTGAACTAGGCGTTTGCTTGAAATGTATTGGCATGAGTGGTACTGGATAGTGCT

181 190 200 210 220 230

181 AGAATCAACTTGATCCGCAAACGAACTTTACATAACCGTACTCACCATGACCTATCACGA

241 ATATGACTTTCAATGTATTAGGTTTATCCAACTCGTTGAATAGTTTAATGGTATATTTCT

241 250 260 270 280 290

241 TATACTGAAAGTTACATAATCCAAATAGGTTGAGCAACTTATCAAATTACCATATAAAGA

301 CGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGA

301 310 320 330 340 350

301 GCCATAAGATCCGAGCCGGAATGTTATATTGTTTGTTCAAACTGGAGTTTAGTCCATCCT

361 TTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCT

361 370 380 390 400 410

361 AATGGGCGACTTGAATTCGTATAGTTATTCGCCTCCTTTTCTTTGGTTGTCCCTAACGGA

421 TAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGCACCTTCGGTGTCCG

421 430 440 450 460 470

421 ATCATTGCCGCTCACTTCGCCGTTTTCGAGTTTAAACTTTAGACCGTGGAAGCCACAGGC

481 AGTTGTAATTTGAAGAAGGTAACTTTGGAGTTGGCTCTTGTCTATGTTCCTTGGAACAGG

481 490 500 510 520 530

481 TCAACATTAAACTTCTTCCATTGAAACCTCAACCGAGAACAGATACAAGGAACCTTGTCC

541 ACGTCACAGAGGGTGAGAATCCCGTGCGATGAGATGCCCAATTCTATGTAAAGTGCTTTC

541 550 560 570 580 590

541 TGCAGTGTCTCCCACTCTTAGGGCACGCTACTCTACGGGTTAAGATACATTTCACGAAAG

601 GAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCATCTAAAGCTA

601 610 620 630 640 650

601 CTTCTCAGCTCAACAAACCCTTACGTCGAGATTCACCCACCATTTAAGGTAGATTTCGAT

661 AATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTG

661 670 680 690 700 710

661 TTATAACCGCTCTCTGGCTATCGCTTGTTCATGTCACTACCTTTCTACTTTTCTTGAAAC

721 AAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCTTGAGATCAGACTTGG

721 730 740 750 760 770

721 TTTTCTCTCACTTTTTCATGCACTTTAACAACTTTCCCTTCCCGAACTCTAGTCTGAACC

<<<Fam-R<<< 800 to 818

781 TATTTTGCGATCCTTTCCTTCTTGGTTGGGTTCCTCGC

781 790 800 810

781 ATAAAACGCTAGGAAAGGAAGAACCAACCCAAGGAGCG

Results for linear 302 residue sequence "*M._guiliermondii*" starting "GGATCTCTTG"

>>>Guil-F>>> 1 to 19

>>>Kef-F>>> 1 to 19

>>>Lusi-F>>> 1 to 19

>>>Fam-F>>> 1 to 19

>>>Rugo-F>>> 1 to 19

1 GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATT

1 10 20 30 40 50

1 CCTAGAGAACCAAGAGCGTAGCTACTTCTTGCGTCGCTTTACGCTATTCATTATACTTAA

61 GCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCAGAG

61 70 80 90 100 110

61 CGTCTAAAAGCACTTAGTAGCTTAGAAACTTGCGTGTAACGCGGGAGACCATAAGGTCTC

121 GGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCCCCGGGTTTGGTATTGAGTGATAC

121 130 140 150 160 170

121 CCGTACGGACAAACTCGCAGTAAAGAGAGAGTTTGGGGGCCCAAACCATAACTCACTATG

181 TCTTAGTCGGACTAGGCGTTTGCTTGAAAAGTATTGGCATGGGTAGTACTAGATAGTGCT

181 190 200 210 220 230

181 AGAATCAGCCTGATCCGCAAACGAACTTTTCATAACCGTACCCATCATGATCTATCACGA

<<<Guil-R<<< 284 to 302

241 GTCGACCTCTCAATGTATTAGGTTTATCCAACTCGTTGAATGGTGTGGCGGGATATTTCT

241 250 260 270 280 290

241 CAGCTGGAGAGTTACATAATCCAAATAGGTTGAGCAACTTACCACACCGCCCTATAAAGA

301 GG

301

301 CC

Results for linear 206 residue sequence "*Kl._marxianus*" starting "GATCTCTTGG"

1 *GATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTTGCGATATGTATTGTGAATT

1 10 20 30 40 50

1 *CTAGAGAACCAAGAGCGTAGCTACTTCTTGCGTCGCTTAAACGCTATACATAACACTTAA

61 GCAGANTTTTCGTGAATCATCAAATTCTTTGAACGCACATTGCGCCCTCTGGTATTNCCA

61 70 80 90 100 110

61 CGTCTNAAAAGCACTTAGTAGTTTAAGAAACTTGCGTGTAACGCGGGAGACCATAANGGT

121 GGGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAACCTTTGGGTTTGGTAGTGAGTGA

121 130 140 150 160 170

121 CCCCCGTACGGACAAACTCGCAGTAAAGAGAGAGTTTGGAAACCCAAACCATCACTCACT

<<<Kef-R<<< 186 to 206

181 TACTCGTCTCGGGTTAACTTGAAAGT

181 190 200

181 ATGAGCAGAGCCCAATTGAACTTTCA

Results for linear 149 residue sequence "*Y._lipolytica*" starting "ACCGAGAGCG"

>>>Lipo-F>>> 1 to 17

1 ACCGAGAGCGACGAGTACGATACGCCCGAGATATCCTGCAGAAGGAACTGCTGCCACACA

1 10 20 30 40 50

1 TGGCTCTCGCTGCTCATGCTATGCGGGCTCTATAGGACGTCTTCCTTGACGACGGTGTGT

61 TCACCCAGGAGGAGGGTTTCGAGACCCGAAAGGCGTACTTCCTGGGCTACATGGTGCATC

61 70 80 90 100 110

61 AGTGGGTCCTCCTCCCAAAGCTCTGGGCTTTCCGCATGAAGGACCCGATGTACCACGTAG

<<<Lipo-R<<< 130 to 149

121 GAATGCTGCTTGTGGCTCTGGGTAGAAAG

121 130 140

121 CTTACGACGAACACCGAGACCCATCTTTC

Results for linear 377 residue sequence "*Cl._lusitaniae*" starting "GGATCTCTTG"

>>>Guil-F>>> 1 to 19

>>>Kef-F>>> 1 to 19

>>>Lusi-F>>> 1 to 19

>>>Fam-F>>> 1 to 19

>>>Rugo-F>>> 1 to 19

1 GGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATACGTAGTATGACTT

1 10 20 30 40 50

1 CCTAGAGAACCAAGAGCGTAGCTACTTCTTGCGTCGCTTAACGCTATGCATCATACTGAA

61 GCAGACGTGAATCATCGAATCTTTGAACGCACATTGCGCCTCGAGGCATTCCTCGAGGCA

61 70 80 90 100 110

61 CGTCTGCACTTAGTAGCTTAGAAACTTGCGTGTAACGCGGAGCTCCGTAAGGAGCTCCGT

121 TGCCTGTTTGAGCGTCGCATCCCCTCTAACCCCCGGTTAGGCGTTGCTCCGAAATATCAA

121 130 140 150 160 170

121 ACGGACAAACTCGCAGCGTAGGGGAGATTGGGGGCCAATCCGCAACGAGGCTTTATAGTT

181 CCGCGCTGTCAAACACGTTTACAGCACGACATTTCGCCCTCAAATCAGGTAGGACTACCC

181 190 200 210 220 230

181 GGCGCGACAGTTTGTGCAAATGTCGTGCTGTAAAGCGGGAGTTTAGTCCATCCTGATGGG

241 GCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAA

241 250 260 270 280 290

241 CGACTTGAATTCGTATAGTTATTCGCCTCCTTTTCTTTGGTTGTCCCTAACGGGGTCATT

<<<Lusi-R<<< 359 to 377

301 CGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCCTGCGGGAATTGTAATTTGAAGGT

301 310 320 330 340 350

301 GCCGCTCACTTCGCCGTTTTCGAGTTTAAACTTTAGGACGCCCTTAACATTAAACTTCCA

361 TTCGTGGTCTGAGTCGG

361 370

361 AAGCACCAGACTCAGCC

Results for linear 537 residue sequence "*P._norvegensis*" starting "GGGTTTGGAA"

>>>Norve-F>>> 1 to 21

1 GGGTTTGGAACCAATCTCAGATTATGATCCACAAGAACATAGGTTATCTACAAGAGTTTT

1 10 20 30 40 50

1 CCCAAACCTTGGTTAGAGTCTAATACTAGGTGTTCTTGTATCCAATAGATGTTCTCAAAA

61 CCTTAATGGTGATTGGGTTGGTACACATAGAGATCCGGGTATGTTAGTTGATACAATGAG

61 70 80 90 100 110

61 GGAATTACCACTAACCCAACCATGTGTATCTCTAGGCCCATACAATCAACTATGTTACTC

121 GCAATTAAGAAGAAGTGGTACTATTTCAGCAGAAGTTTCCTTAATTAGAGATATTAGAGA

121 130 140 150 160 170

121 CGTTAATTCTTCTTCACCATGATAAAGTCGTCTTCAAAGGAATTAATCTCTATAATCTCT

181 AAGAGAATTTAAGATTTTCACTGATGCTGGTAGAGTTTATAGACCATTATTCATTGTTGA

181 190 200 210 220 230

181 TTCTCTTAAATTCTAAAAGTGACTACGACCATCTCAAATATCTGGTAATAAGTAACAACT

241 TGATGATCCAGATTCTCCAACTAAGGGAGGTTTGAAATTAACTAAAGAACATTGTAGGAA

241 250 260 270 280 290

241 ACTACTAGGTCTAAGAGGTTGATTCCCTCCAAACTTTAATTGATTTCTTGTAACATCCTT

301 GATTCTTGATAGAGAAATTGAAGAGATACCACAAGATGATGGATATGATGAAAATGGAGC

301 310 320 330 340 350

301 CTAAGAACTATCTCTTTAACTTCTCTATGGTGTTCTACTACCTATACTACTTTTACCTCG

361 AGCATTGGAACCAATACAGAGAATTTATGGTTGGGACTCATTATTGAGTGAAGGTGTTGT

361 370 380 390 400 410

361 TCGTAACCTTGGTTATGTCTCTTAAATACCAACCCTGAGTAATAACTCACTTCCACAACA

421 TGAATATTTGGATGTTGAGGAAGAAGAGACTGTTTTAATTGCCATGTCATCAGAAGATTT

421 430 440 450 460 470

ACTTATAAACCTACAACTCCTTCTTCTCTGACAAAATTAACGGTACAGTAGTCTTCTAAA

<<<Norve-R<<< 517 to 537

481 ATCAATGNATGATGAAGATGAAATGGATGAANATGACAGCGAAAACACCGATTTTGC

481 490 500 510 520 530

481 TAGTTACNTACTACTTCTACTTTACCTACTTNTACTGTCGCTTTTGTGGCTAAAACG

Results for linear 689 residue sequence "*D._rugose*" starting "GGATCTCTAG"

*

1 GGATCTCTAGGTTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTACGAAAC

1 10 20 30 40 50

1 CCTAGAGATCCAAGAGCGTAGCTACTTCTTGCGTCGCTTTACGCTATGCATCATGCTTTG

61 GCAAGTCGTGAATCATCGAATCTTTGAACGCACATTGCGCTGTGTGGCATTCCGCACAGC

61 70 80 90 100 110

61 CGTTCAGCACTTAGTAGCTTAGAAACTTGCGTGTAACGCGACACACCGTAAGGCGTGTCG

121 ATGCCTGTTTGAGCAATATTTCTCTCTCGCAAGGTGTTGGGCACCACGCCGGCAGGCGTC

121 130 140 150 160 170

121 TACGGACAAACTCGTTATAAAGAGAGAGCGTTCCACAACCCGTGGTGCGGCCGTCCGCAG

181 TGCCCGAAACGCGACCGTCTAAAACAGTTAAGCTTGTTACAGACTCACGATCTTATTCTC

181 190 200 210 220 230

181 ACGGGCTTTGCGCTGGCAGATTTTGTCAATTCGAACAATGTCTGAGTGCTAGAATAAGAG

241 AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCA

241 250 260 270 280 290

241 TTTAGTCCATCCTGATGGGCGACTTGAATTCGTATAGTTATTCGCCTCCTTTTCTTTGGT

301 ACCGGGATTGCCTCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAAGCCCGCG

301 310 320 330 340 350

301 TGGCCCTAACGGAGTCATTGCCGCTCACTTCGCCGTTGTCGAGTTTAAACTTTCGGGCGC

361 GGCGTTGTAATTTGCAGGCGGATGTTTTGGGGCGGGCGCTGTCTACGTTCCTTGGAACAG

361 370 380 390 400 410

361 CCGCAACATTAAACGTCCGCCTACAAAACCCCGCCCGCGACAGATGCAAGGAACCTTGTC

421 GACGCCGCAGAGGGTGAGAGCCCCGTGCGATGGCGCCTCCAACCGCGTAAAACTCCGCCG

421 430 440 450 460 470

421 CTGCGGCGTCTCCCACTCTCGGGGCACGCTACCGCGGAGGTTGGCGCATTTTGAGGCGGC

481 ACGAGTCGAGTTGTTTGGGAATGCAGCTCCAAGTGGGTGGTAAATTCCATCTAAAGCTAA

481 490 500 510 520 530

481 TGCTCAGCTCAACAAACCCTTACGTCGAGGTTCACCCACCATTTAAGGTAGATTTCGATT

541 ATACTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGCACTTTGA

541 550 560 570 580 590

541 TATGACCGCTCTCTGGCTATCGCTTGTTCATGTCACTACCTTTCTACTTTTCGTGAAACT

601 AAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATGCGATTAGCGGCCAG

601 610 620 630 640 650

601 TTTCTCTCACTTTGTCGTGCACTTTAACAACTTTCCCTTCCCATACGCTAATCGCCGGTC

<<<Rugo-R<<< 670 to 689

661 CAGGAGGTGCCTTCTCGTGAAAAGGCCGT

661 670 680

661 GTCCTCCACGGAAGAGCACTTTTCCGGCA

Results for linear 235 residue sequence "*Cryptococcus_deneoformans*" starting "CCATCCTGCT"

*

1 CCATCCTGCTGGCGAAGATGCAGTCAGCAACCAAAAAATTCTGGTAAGCACTCTGAAACG

1 10 20 30 40 50

1 GGTAGGACGACCGCTTCTACGTCAGTCGTTGGTTTTTTAAGACCATTCGTGAGACTTTGC

61 ACTTGGGCGAGTCTCAGGATGCATGAGAGGCTTGCCGATTCACAATTTTATCGAGCAGGC

61 70 80 90 100 110

61 TGAACCCGCTCAGAGTCCTACGTACTCTCCGAACGGCTAAGTGTTAAAATAGCTCGTCCG

121 GATGTTTCCTTCATTCCATTTCATCTTTCAGCTGGCCTTAGGATACGGCAGAACAAGACA

121 130 140 150 160 170

121 CTACAAAGGAAGTAAGGTAAAGTAGAAAGTCGACCGGAATCCTATGCCGTCTTGTTCTGT

<<<Deneo-R<<< 215 to 235

<<<Neo-R<<< 215 to 235

<<<Gattii-R<<< 215 to 235

181 AGTAGGGAAGTTAGTGTTATAATCTTATCCACGGCCATAGCCTTCATACAGCACC

181 190 200 210 220 230

181 TCATCCCTTCAATCACAATATTAGAATAGGTGCCGGTATCGGAAGTATGTCGTGG

Results for linear 392 residue sequence "*Cryptococcus_neoformans*" starting "CCATCCTGTT"

>>>Deneo-F>>> 1 to 18

>>>Neo-F>>> 1 to 18

1 CCATCCTGTTGGCGAAGATGCAATCACCAACAAAAAAATTTGGTAAGCACCCTGAACCGA

1 10 20 30 40 50

1 GGTAGGACAACCGCTTCTACGTTAGTGGTTGTTTTTTTAAACCATTCGTGGGACTTGGCT

61 CTTGAGGGAGTGTCAGGATGCATGAGCAGACTCAGCCTTTTATCTTCGGCCTCACTGGCA

61 70 80 90 100 110

61 GAACTCCCTCACAGTCCTACGTACTCGTCTGAGTCGGAAAATAGAAGCCGGAGTGACCGT

121 CACGCTTGAAGATCAGAGGCAGTCATATAACCCTGTCCCCATTATCAGCTTGAGAACACG

121 130 140 150 160 170

121 GTGCGAACTTCTAGTCTCCGTCAGTATATTGGGACAGGGGTAATAGTCGAACTCTTGTGC

181 GGCACTCTTGCACGTCAAAAAAATGTACCCATTGGAAGCGGCGCTAGGGCCAAGCGGCTT

181 190 200 210 220 230

181 CCGTGAGAACGTGCAGTTTTTTTACATGGGTAACCTTCGCCGCGATCCCGGTTCGCCGAA

241 GTCGCTGTCAATCTTATCGACAGGCTGGTGTTTCCTTCGTTCCCTTTCGTCTTTCAACTC

241 250 260 270 280 290

241 CAGCGACAGTTAGAATAGCTGTCCGACCACAAAGGAAGCAAGGGAAAGCAGAAAGTTGAG

301 GTCTTAGGAAGGAAACAAGATAAAGTAGGACAAGACAAGTAGGGAGGTTAGTGTTATAAT

301 310 320 330 340 350

301 CAGAATCCTTCCTTTGTTCTATTTCATCCTGTTCTGTTCATCCCTCCAATCACAATATTA

<<<Deneo-R<<< 372 to 392

<<<Neo-R<<< 372 to 392

<<<Gattii-R<<< 372 to 392

361 CTTATCCACGGCCATAGCCTTCATACAGCACC

Results for linear 184 residue sequence "*Cryptococcus_gattii*" starting "CAGGAGCGGA"

*

1 CAGGAGCGGATTCAGCGTTTTCTTTGGCCTTACTTATCGAGGGATTCGGTGTCTCCTTCA

1 10 20 30 40 50

1 GTCCTCGCCTAAGTCGCAAAAGAAACCGGAATGAATAGCTCCCTAAGCCACAGAGGAAGT

61 TTCCCTTTTCGTTTTTCAACCGGTCATAGAAACAAGATTCTTAAGACGTGATTTGATTAG

61 70 80 90 100 110

61 AAGGGAAAAGCAAAAAGTTGGCCAGTATCTTTGTTCTAAGAATTCTGCACTAAACTAATC

<<<Deneo-R<<< 164 to 184

<<<Neo-R<<< 164 to 184

<<<Gattii-R<<< 164 to 184

121 AACAAGACAAGTAGGGAAATTAGTGTTATGTTGTTATCCACGGCCATAGCCTTCATACAG

121 130 140 150 160 170

121 TTGTTCTGTTCATCCCTTTAATCACAATACAACAATAGGTGCCGGTATCGGAAGTATGTC

181 CACC

181

181 GTGG

Results for linear 299 residue sequence "*Geotrichum_candidum*" starting "AGATTGTATC"

>>>Gcandi-F>>> 1 to 24

1 AGATTGTATCTTGAGAGCGGATTAAAGTCTGTTGGAACACAGCGCCTTAGAGGGTGACAG

1 10 20 30 40 50

1 TCTAACATAGAACTCTCGCCTAATTTCAGACAACCTTGTGTCGCGGAATCTCCCACTGTC

61 CCCCGTAAAATCTATTCTCATTGTAAGATACTTTCGAAGAGTCGAGTTGTTTGGGAATGC

61 70 80 90 100 110

61 GGGGCATTTTAGATAAGAGTAACATTCTATGAAAGCTTCTCAGCTCAACAAACCCTTACG

121 AGCTCTAAGTGGGAGGTAAATTCCTTCTAAAGCTAAATATTGACGAGAGACCGATAGCGA

121 130 140 150 160 170

121 TCGAGATTCACCCTCCATTTAAGGAAGATTTCGATTTATAACTGCTCTCTGGCTATCGCT

181 ACAAGTACTGTGAAGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAA

181 190 200 210 220 230

181 TGTTCATGACACTTCCTTTCTACTTTTCGTGAAACTTTTCTCTCACTTTTTCATGCACTT

241 ATTGTTAAAAGGGAAGGGTATTGAATCAGACTTGGTGCTGTTGTTCAACTGTGTTTTGG

241 250 260 270 280 290 ***

241 TAACAATTTTCCCTTCCCATAACTTAGTCTGAACCACGACAACAAGTTGACACAAAACC

Results for linear 113 residue sequence "*Rhodotorula_mucliginosa*" starting "GTCTAGCTCG"

*

1 GTCTAGCTCGTTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGCGTAA

1 10 20 30 40 50

1 CAGATCGAGCAAGCATTACGTAATCGTAGGCGTTAGCTTGAAGCCTAACTGAACCGCATT

<<<Rhodo-R<<< 96 to 113

61 TAGACTATTCGCTGAGGAATTCTAGTCTTCGGATTAGAGCCGGGTTGGGTTAA

61 70 80 90 100 110

61 ATCTGATAAGCGACTCCTTAAGATCAGAAGCCTAATCTCGGCCCAACCCAATT

Results for linear 483 residue sequence "*Trichosporon_asahii*" starting "GAAGGATCAT"

>>>Tasahii-F>>> 1 to 23

>>>Tlactis-F>>> 1 to 23

1 GAAGGATCATTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAAC

1 10 20 30 40 50

1 CTTCCTAGTAATCACTAACGGAAATATCCGAATATTGATATAGGTGAATGTGGACACTTG

61 TGTTCTACTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTT

61 70 80 90 100 110

61 ACAAGATGATGAACTGCGTTCAGCTCATAAAAATGTTTGTTACACATTACTTGCAGCAAA

121 TATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAAC

121 130 140 150 160 170

121 ATAATATTGTTTTATTTTGAAAGTTGTTGCCTAGAGAACCGAGAGCGTAGCTACTTCTTG

181 GCAGCGAATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA

181 190 200 210 220 230

181 CGTCGCTTAACGCTATTCATTACACTTAACGTCTTAAGTCACTTAGTAGCTTAGAAACTT

241 CGCAGCTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTCAGTGTCATGAAATCTCA

241 250 260 270 280 290

241 GCGTCGAACGCGAGAGACCATAAGGCCTCTCGTACGGACAAAGTCACAGTACTTTAGAGT

301 ACCACTAGGGTTTCCTAATGGATTGGATTTGGGCGTCTGCGATTTCTGATCGCTCGCCTT

301 310 320 330 340 350

301 TGGTGATCCCAAAGGATTACCTAACCTAAACCCGCAGACGCTAAAGACTAGCGAGCGGAA

361 AAAAGAGTTAGCAAGTTTGACATTAATGTCTGGTGTAATAAGTTTCACTGGGTCCATTGT

361 370 380 390 400 410

361 TTTTCTCAATCGTTCAAACTGTAATTACAGACCACATTATTCAAAGTGACCCAGGTAACA

<<<Tasahii-R<<< 464 to 483

<<<Tlactis-R<<< 464 to 483

421 GTTGAAGCGTGCTTCTAATCGTCCGCAAGGACAATTACTTTGACTCTGGCCTGAAATCAG

421 430 440 450 460 470

421 CAACTTCGCACGAAGATTAGCAGGCGTTCCTGTTAATGAAACTGAGACCGGACTTTAGTC

481 GTA

481

481 CAT

Results for linear 480 residue sequence "*Trichosporon_lactis*" starting "GAAGGATCAT"

>>>Tasahii-F>>> 1 to 23010

>>>Tlactis-F>>> 1 to 23

1 GAAGGATCATTAGTGATTGCCTTTATAGGCTTAACTATATCCACATACACCTGTGAACTG

1 10 20 30 40 50

1 CTTCCTAGTAATCACTAACGGAAATATCCGAATTGATATAGGTGTATGTGGACACTTGAC

61 TTCTACTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGTTTTA

61 70 80 90 100 110

61 AAGATGATGAACTGCGTTCAGCTCATAAAAATGTTTGTTACACATTACTTGCAGCAAAAT

121 TTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGC

121 130 140 150 160 170

121 AATATTGTTTTATTTTGAAAGTTGTTGCCTAGAGAACCGAGAGCGTAGCTACTTCTTGCG

181 AGCGAATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG

181 190 200 210 220 230

181 TCGCTTAACGCTATTCATTACACTTAACGTCTTAAGTCACTTAGTAGCTTAGAAACTTGC

241 CAGCTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTCAGTGTCATGAAATCTCAAC

241 250 260 270 280 290

241 GTCGAACGCGAGAGACCATAAGGCCTCTCGTACGGACAAAGTCACAGTACTTTAGAGTTG

301 CACTAGGGTTTCCTAATGGATTGGATTTGGGTGTTGCGATCTCTGATCGCTCGCCTTAAA

301 310 320 330 340 350

301 GTGATCCCAAAGGATTACCTAACCTAAACCCACAACGCTAGAGACTAGCGAGCGGAATTT

361 AGAGTTAGCAAGTTTGACATATATGTCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTT

361 370 380 390 400 410

361 TCTCAATCGTTCAAACTGTATATACAGACCACATTATTCAAAGTGACCCAGGTAACACAA

<<<Tasahii-R<<< 461 to 480

<<<Tlactis-R<<< 461 to 480

421 GAAGCGTGCTTCTAATCGTCCGCAAGGACAATTACTTTGACTCTGGCCTGAAATCAGGTA

421 430 440 450 460 470

421 CTTCGCACGAAGATTAGCAGGCGTTCCTGTTAATGAAACTGAGACCGGACTTTAGTCCAT

Chapter 3: Application of a wastewater surveillance system to the detection of pathogenic yeast

(Parts of this chapter have been submitted for publication)

Molecular identification using ITS primers

Table S1 Yeast species identified over 12 months with the percentage identification according to NCBI's BLAST database

Number	Yeast identified	Percentage ID
May 2023		
UFS 1.1	*Clavispora lusitaniae*	97.52
UFS 1.2	*Clavispora lusitaniae*	95.12
UFS 1.3	*Clavispora lusitaniae*	95.6
UFS 1.4	*Candida albicans*	100
UFS 1.5	*Meyerozyma guilliermondii*	100
UFS 1.6	*Clavispora lusitaniae*	99.15
BV 1.1	*Candida glabrata*	100
BV 1.2	*Saccharomyces cerevisiae*	99.8
BV 1.3	*Candida albicans*	99.82
BV 1.4	*Sample was a bacterium*	
BV 1.5	*Clavispora lusitaniae*	98.66
BV 1.6	*Candida glabrata*	100
WL 1.1	*Candida albicans*	99.1
WL 1.2	*Candida palmioleophila*	99.7
WL 1.3	*Candida albicans*	93.53
WL 1.4	*Reverse sequence not complete*	
WL 1.5	*Candida glabrata*	99.09
WL 1.6	*Candida glabrata*	82.9
BW 1.1	*Saccharomyces cerevisiae*	99.75
BW 1.2	*Candida glabrata*	99.88
BW 1.3	*Saccharomyces cerevisiae*	99.88

BW 1.4	*Candida glabrata*	100
BW 1.5	*Candida glabrata*	99.89
BW 1.6	*Candida glabrata*	99.59
SW not working		
NNE 1.1	*Pichia kudriavzevii*	98.06
NNE 1.2	*Pichia kudriavzevii*	99.61
NNE 1.3	*Candida glabrata*	99.44
NNE 1.4	*Candida glabrata*	99.77
NNE 1.5	*Candida glabrata*	98.4
NNE 1.6	*Saccharomyces cerevisiae*	99.16
July 2023		
UFS 3.1	*Candida tropicalis*	100
UFS 3.2	*Candida albicans*	99.8
UFS 3.3	*Saccharomyces cerevisiae*	99.39
UFS 3.4	*Saccharomyces cerevisiae*	99.63
UFS 3.5	*Saccharomyces cerevisiae*	99.32
UFS 3.6	*Saccharomyces cerevisiae*	99.62
BV 3.1	*Candida tropicalis*	99.58
BV 3.2	*Pichia cactophila (forward only)*	87.62
BV 3.3	*Saccharomyces cerevisiae*	97.88
BV 3.4	*Candida glabrata*	99.65
BV 3.5	*Candida glabrata*	99.88
BV 3.6	*Candida glabrata*	98.25
WL 3.1	*Candida albicans*	100
WL 3.2	*Pichia kudriavzevii*	100
WL 3.3	*Saccharomyces cerevisiae*	97.63
WL 3.4	*Candida palmioleophila*	99.66
WL 3.5	*Candida albicans*	99.81
WL 3.6	*Sporopachydermia lactativora*	98.37
BW 3.1	*[Candida] glabrata*	99.64
BW 3.2	*Saccharomyces cerevisiae*	97.13
BW 3.3	*Candida tropicalis*	100

BW 3.4	*Saccharomyces cerevisiae*	99.44
BW 3.5	*Candida glabrata*	99.41
BW 3.6	*Saccharomyces cerevisiae*	96.43
SW Could not isolate		
NNE 3.1	*Pichia kudriavzevii*	100
NNE 3.2	*Candida glabrata*	99.46
NNE 3.3	*Sample was a bacterium*	
NNE 3.4	*Candida stellimalicola*	99.21
NNE 3.5	*Pichia kudriavzevii*	98.53
NNE 3.6	*Pichia kudriavzevii*	100
September 2023		
UFS 5.1	*Candida glabrata*	99.63
UFS 5.2	*Saccharomyces cerevisiae*	99.03
UFS 5.3	*Saccharomyces cerevisiae*	99.63
UFS 5.4	*Hanseniaspora pseudoguilliermondii*	99.86
UFS 5.5	*Candida albicans*	100
UFS 5.6	*Saccharomyces cerevisiae*	99.59
BV 5.1	*Candida albicans*	100
BV 5.2	*Candida glabrata*	99.63
BV 5.3	*Saccharomyces cerevisiae*	99.17
BV 5.4	*Saccharomyces cerevisiae (forward sequence only)*	86.8
BV 5.5	*Candida albicans*	99.63
BV 5.6	*Candida albicans*	99.82
WL 5.1	*Candida albicans*	99.63
WL 5.2	*Candida glabrata*	99.64
WL 5.3	*Pichia kudriavzevii*	99.61
WL 5.4	*Candida glabrata (Revers sequence only)*	97.06
WL 5.5	*Candida glabrata*	99.65
WL 5.6	*Pichia kudriavzevii*	100
BW 5.1	*Candida glabrata*	100
BW 5.2	*Saccharomyces cerevisiae*	99.6

BW 5.3		
BW 5.4	*[Candida] sp. (uncertain placement)*	100
BW 5.5	*Candida tropicalis*	99.81
BW 5.6	*Saccharomyces cerevisiae*	99.87
SW 5.1	*Saccharomyces cerevisiae*	99.87
SW 5.2	*Pichia kudriavzevii*	99.62
SW 5.3	*[Candida] sp. (uncertain placement)*	100
SW 5.4		
SW 5.5	*Saccharomyces cerevisiae*	99.42
SW 5.6		
NNE 5.1	*Candida glabrata*	99.66
NNE 5.2	*Pichia kudriavzevii*	97.64
NNE 5.3	*Pichia kudriavzevii*	99.8
NNE 5.4	*Saccharomyces cerevisiae*	99.64
NNE 5.5	*Candida glabrata*	99.5
NNE 5.6	*Candida albicans (forward sequence only)*	100
November 2023		
UFS 7.1	*Saccharomyces cerevisiae*	99.64
UFS 7.2	*Saccharomyces cerevisiae*	100
UFS 7.3		
UFS 7.4	*Magnusiomyces capitatus*	100
UFS 7.5	*Saccharomyces cerevisiae*	99.51
UFS 7.6	*Saccharomyces cerevisiae*	99.87
BV not working		
WL 7.1	*Pichia kudriavzevii*	99.08
WL 7.2	*Saprochaete clavata*	99.79
WL 7.3	*Pichia kudriavzevii (forward sequence only)*	93.11
WL 7.4	*Pichia kudriavzevii*	100
WL 7.5	*Sporopachydermia lactativora*	99.84
WL 7.6	*Pichia kudriavzevii (forward sequence only)*	94.62
BW 7.1		
BW 7.2		

BW 7.3	*Saccharomyces cerevisiae*	99.63
BW 7.4	*Dipodascus capitatus*	96.33
BW 7.5	*Sample was a bacterium*	
BW 7.6	*Exophiala dermatitidis*	100
SW 7.1	*Pichia kudriavzevii*	99.8
SW 7.2	*Pichia kudriavzevii*	99.8
SW 7.3	*Pichia kudriavzevii*	99.8
SW 7.4	*Pichia kudriavzevii*	99.8
SW 7.5	*Pichia kudriavzevii*	99.8
SW 7.6	*Pichia kudriavzevii*	100
NNE 7.1	*Candida glabrata*	96.06
NNE 7.2	*Pichia kudriavzevii*	99.8
NNE 7.3	*Candida glabrata (revers sequence only)*	92.31
NNE 7.4	*Candida glabrata (reverse sequence only)*	93.54
NNE 7.5	*Pichia kudriavzevii*	98.8
NNE 7.6	*Pichia kudriavzevii*	100
January 24		
UFS not working		
BV 9.1	*Sample was a bacterium*	
BV 9.2	*Pichia sporocuriosa (forward sequence only)*	94.56
BV 9.3	*Candida tropicalis*	99.05
BV 9.4	*Pichia kudriavzevii (reverse sequence only)*	83.41
BV 9.5	*Candida tropicalis*	99.92
BV 9.6	*Pichia kudriavzevii (forward sequence only)*	93.1
WL 9.1	*Pichia kudriavzevii*	99.6
WL 9.2	*Candida glabrata*	100
WL 9.3	*Candida glabrata*	100
WL 9.4	*Pichia kudriavzevii*	100
WL 9.5	*Candida albicans*	100
WL 9.6	*Pichia kudriavzevii*	100
BW 9.1	*Saccharomyces cerevisiae*	99.52
BW 9.2	*Candida parapsilosis*	100

BW 9.3	*Candida albicans*	99.82
BW 9.4	*Sample was a bacterium*	
BW 9.5	*Saccharomyces cerevisiae*	99.76
BW 9.6	*Clavispora lusitaniae*	100
SW 9.1	*Candida tropicalis*	100
SW 9.2	*Candida glabrata*	100
SW 9.3	*Sample was a bacterium*	
SW 9.4	*Saccharomyces cerevisiae*	99.4
SW 9.5	*Pichia kudriavzevii*	100
SW 9.6		
NNE 9.1	*Pichia kudriavzevii*	100
NNE 9.2	*Pichia kudriavzevii (forward sequence only)*	94.29
NNE 9.3	*Pichia kudriavzevii*	100
NNE 9.4	*Pichia kudriavzevii*	100
NNE 9.5	*Pichia kudriavzevii*	98.68
NNE 9.6	*Candida tropicalis (reverse sequence only)*	100
March 2024		
UFS not working		
BV 11.1	*Sample was a bacterium*	
BV 11.2	*Candida glabrata*	100
BV 11.3	*Pichia kudriavzevii*	100
BV 11.4	*Candida glabrata*	100
BV 11.5	*Pichia kudriavzevii*	100
BV 11.6	*Candida glabrata*	100
WL 11.1	*Pichia kudriavzevii*	100
WL 11.2	*Candida albicans*	100
WL 11.3	*Candida albicans*	99.82
WL 11.4	*Candida albicans*	100
WL 11.5	*Candida glabrata*	99.89
WL 11.6	*Pichia kudriavzevii*	100
BW 11.1	*Pichia kudriavzevii*	99.8
BW 11.2	*Sample was a bacterium*	

BW 11.3	*Candida glabrata*	100
BW 11.4	*Magnusiomyces capitatus*	100
BW 11.5	*Sample was a bacterium*	
BW 11.6	*Pichia kudriavzevii*	99.8
SW 11.1	*Candida glabrata*	99.65
SW 11.2	*Sample was a bacterium*	
SW 11.3		
SW 11.4	*Pichia kudriavzevii*	100
SW 11.5	*Candida glabrata*	100
SW 11.6	*Pichia kudriavzevii*	100
NNE 11.1	*Candida glabrata*	100
NNE 11.2	*Candida glabrata*	99.19
NNE 11.3	*Candida glabrata*	100
NNE 11.4	*Pichia kudriavzevii*	100
NNE 11.5	*Pichia kudriavzevii*	100
NNE 11.6	*Sample was a bacterium*	

Acquisition of hospital data on fungal infections

Table S2 Public hospital data on fungal infections from May 2023 to April 2024

May 2023					
Hospital	Date	Sample	Method	Growth	ID
Universitas hospital	5/2/2023	Tissue	Culture mycology	N	
Universitas hospital	5/3/2023	Tissue	Culture mycology	N	
Pelonomi hospital	5/4/2023	Blood culture	Culture mycology	N	
Universitas hospital	5/5/2023	Tissue	Culture mycology	N	

Universitas hospital	5/5/2023	Tissue	Culture mycology	N	
Universitas hospital	5/5/2023	Tissue	Culture mycology	N	
Universitas hospital annex	5/5/2023	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	5/6/2023	Skin tissue	Culture mycology	N	
Universitas hospital	5/6/2023	Fluid / aspirate	Culture mycology	Y	
Universitas hospital	5/6/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	5/6/2023	Tissue	Culture mycology	N	
Universitas hospital	5/8/2023	Tissue	Culture mycology	Y	*Fusarium* species
Pelonomi hospital	5/8/2023	Tissue	Culture mycology	N	
NHLS qa free state academic (ce)	5/8/2023	Slide	Culture mycology	Y	YEAST
NHLS qa free state academic (ce)	5/8/2023	Blood culture	Culture mycology	Y	*Candida glabrata*
NHLS qa free state academic (ce)	5/8/2023	Blood culture	Culture mycology	Y	*Candida krusei*
NHLS qa free state academic (ce)	5/8/2023	Tissue	Culture mycology	Y	*Acremonium* species
NHLS qa free state academic (ce)	5/8/2023	Tissue	Culture mycology	Y	*Bipolaris* species
NHLS qa free state academic (ce)	5/8/2023	Bal	Culture mycology	Y	*Cunninghamella* species
NHLS qa free state academic (ce)	5/8/2023	Tissue	Culture mycology	Y	*Gliocladium* species

Universitas hospital	5/8/2023	Tissue	Culture mycology	N	
Universitas hospital	5/9/2023	Sputum	Culture mycology	N	
Universitas hospital	5/9/2023	Blood culture	Culture mycology	N	
Universitas hospital	5/9/2023	Blood culture	Culture mycology	N	
Universitas hospital	5/10/2023	Bronchial washing	Culture mycology	N	
Universitas hospital	5/11/2023	Bctb	Culture mycology	N	
Universitas hospital	5/16/2023	Skin tissue	Culture mycology	N	
Universitas hospital	5/16/2023	Skin tissue	Culture mycology	N	
Universitas hospital	5/16/2023	Tissue	Culture mycology	N	
Universitas hospital	5/16/2023	Tissue	Culture mycology	N	
Universitas hospital	5/18/2023	Tissue	Culture mycology	N	
Universitas hospital	5/19/2023	Tissue	Culture mycology	N	
Universitas hospital	5/20/2023	Peritoneal dialysis fluid	Culture mycology	N	
Universitas hospital	5/22/2023	Tissue	Culture mycology	N	
Universitas hospital	5/22/2023	Tissue	Culture mycology	N	
Universitas hospital	5/22/2023	Tissue	Culture mycology	N	

NHLS qa free state academic (ce)	5/22/2023	Tissue	Culture mycology	Y	*Penicillium* species
NHLS qa free state academic (ce)	5/22/2023	Tissue	Culture mycology	Y	*Curvularia* species
NHLS qa free state academic (ce)	5/22/2023	Tissue	Culture mycology	Y	*Aspergillus flavus*
NHLS qa free state academic (ce)	5/22/2023	Tissue	Culture mycology	Y	*Trichophyton* species
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Universitas hospital	5/23/2023	Tissue	Culture mycology	N	
Schweizer reneke hospital	5/23/2023	Sputum	Culture mycology	N	

Universitas hospital	5/23/2023	Sputum	Culture mycology	N	
Universitas hospital	5/24/2023	Tissue	Culture mycology	N	
Universitas hospital	5/25/2023	Skin tissue	Culture mycology	N	
Universitas hospital	5/25/2023	Swab (superficial)	Culture mycology	N	
Universitas hospital	5/26/2023	Tissue	Culture mycology	N	
NHLS qa free state academic (ce)	5/29/2023	Abscess (superficial) aspirate	Culture mycology	N	
June 2023					
Universitas hospital	6/5/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	6/7/2023	Sputum	Culture mycology	N	
Universitas hospital	6/8/2023	Tissue	Culture mycology	Y	
Universitas hospital	6/8/2023	Tissue	Culture mycology	N	
Universitas hospital	6/9/2023	SKIN TISSUE	Culture mycology	N	
Universitas hospital	6/9/2023	Skin tissue	Culture mycology	N	
Universitas hospital	6/14/2023	Tissue	Culture mycology	N	
Universitas hospital	6/14/2023	Tissue	Culture mycology	N	
Universitas hospital	6/14/2023	Tissue	Culture mycology	N	

Pelonomi hospital	6/14/2023	Bone marrow aspirate	Culture mycology	N	
Pelonomi hospital	6/14/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/14/2023	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	6/14/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/14/2023	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	6/14/2023	Tissue	Culture mycology	N	
Universitas hospital	6/14/2023	Tissue	Culture mycology	N	
Universitas hospital	6/14/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital	6/14/2023	Bronchial alveolar lavage	Culture mycology	N	
Universitas hospital	6/14/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	6/15/2023	Skin tissue	Culture mycology	N	
Pelonomi hospital	6/15/2023	Skin tissue	Culture mycology	N	
Botshabelo hospital	6/15/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital annex	6/15/2023	Fluid / aspirate	Culture mycology	N	
National district hospital	6/17/2023	Midstream urine	Culture mycology	Y	*Geotrichum* species
Universitas hospital	6/18/2023	Tissue	Culture mycology	N	

Universitas hospital	6/18/2023	Tissue	Culture mycology	N	
Universitas hospital	6/19/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/20/2023	Tissue	Culture mycology	N	
Universitas hospital	6/20/2023	Blood culture	Culture mycology	N	
Universitas hospital	6/21/2023	Bronchial washing	Culture mycology	Y	*Candida albicans*
Universitas hospital	6/21/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital annex	6/21/2023	Swab (superficial)	Culture mycology	Y	*Penicillium* species
Universitas hospital annex	6/21/2023	Corneal scraping	Culture mycology	Y	*Penicillium* species
Universitas hospital	6/22/2023	Tissue	Culture mycology	Y	*Aspergillus vesicolor*
Universitas hospital	6/22/2023	Peritoneal dialysis fluid	Culture mycology	N	
Universitas hospital	6/22/2023	Peritoneal dialysis fluid	Culture mycology	N	
Universitas hospital	6/23/2023	Tissue	Culture mycology	N	
Universitas hospital	6/23/2023	Tissue	Culture mycology	N	
Universitas hospital	6/23/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	6/24/2023	Fluid / aspirate	Culture mycology	N	

Pelonomi hospital	6/24/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/27/2023	Tissue	Culture mycology	N	
Pelonomi hospital	6/27/2023	Tissue	Culture mycology	N	
Universitas hospital	6/27/2023	Tissue	Culture mycology	N	
Universitas hospital annex	6/28/2023	Corneal scrapings	Culture mycology	Y	*Penicillium* species
Universitas hospital	6/30/2023	Tissue	Culture mycology	N	
Universitas hospital	6/30/2023	Tissue	Culture mycology	N	
Universitas hospital	6/30/2023	Tissue	Culture mycology	N	
Universitas hospital	6/30/2023	Tissue	Culture mycology	Y	*Scopulariopsis* species
Universitas hospital annex	6/30/2023	Corneal scraping	Culture mycology	Y	*Penicillium* species
July 2023					
Pelonomi hospital	7/1/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/1/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/1/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/1/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/3/2023	Sputum	Culture mycology	N	

Pelonomi hospital	7/4/2023	Tissue	Culture mycology	N	
Universitas hospital	7/5/2023	Tissue	Culture mycology	Y	*Trichoderma* species
Universitas hospital	7/5/2023	Tissue	Culture mycology	N	
Universitas hospital	7/5/2023	Tissue	Culture mycology	N	
Universitas hospital	7/5/2023	Tissue	Culture mycology	N	
Universitas hospital	7/5/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital	7/6/2023	Swab (superficial)	Culture mycology	N	
Universitas hospital annex	7/7/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	7/10/2023	Nail	Culture mycology	Y	*Candida* species
Universitas hospital	7/10/2023	Bronchial alveolar lavage	Culture mycology	Y	*Candida* species
Universitas hospital annex	7/11/2023	Corneal scraping	Culture mycology	N	
Universitas hospital annex	7/11/2023	Corneal scraping	Culture mycology	N	
Pelonomi hospital	7/12/2023	Tissue	Culture mycology	N	
Universitas hospital	7/12/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital annex	7/12/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	7/12/2023	Sputum	Culture mycology	N	

Universitas hospital	7/12/2023	Bronchial alveolar lavage	Culture mycology	N	
Universitas hospital	7/13/2023	Swab (superficial)	Culture mycology	N	
Pelonomi hospital	7/15/2023	Tissue	Culture mycology	N	
Universitas hospital	7/17/2023	Tissue	Culture mycology	N	
Universitas hospital	7/18/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/19/2023	Tissue	Culture mycology	N	
Pelonomi hospital	7/19/2023	Tissue	Culture mycology	N	
Universitas hospital	7/19/2023	Tissue	Culture mycology	N	
Universitas hospital	7/20/2023	Tissue	Culture mycology	N	
Universitas hospital	7/24/2023	Sputum	Culture mycology	N	
Universitas hospital annex	7/25/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	7/25/2023	Sputum	Culture mycology	Y	*Candida* species
Pelonomi hospital	7/26/2023	Tissue	Culture mycology	N	
Universitas hospital annex	7/26/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	7/27/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital annex	7/27/2023	Fluid / aspirate	Culture mycology	N	

Universitas hospital	7/27/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	7/28/2023	Tissue	Culture mycology	N	
Universitas hospital	7/29/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	7/29/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	7/29/2023	Tissue	Culture mycology	N	
NHLS qa free state academic (ce)	7/31/2023	Skin scraping	Culture mycology	Y	YST
NHLS qa free state academic (ce)	7/31/2023	Blood culture	Culture mycology	Y	*Candida* lusitaniae
NHLS qa free state academic (ce)	7/31/2023	CSF	Culture mycology	Y	*Candida auris*
August 2023					
Universitas hospital	8/1/2023	Tissue	Culture mycology	N	
John daniel newberry hospital	8/1/2023	CSF	Culture mycology	N	
Universitas hospital	8/2/2023	Tissue	Culture mycology	N	
Universitas hospital	8/2/2023	Tissue	Culture mycology	N	
Universitas hospital	8/2/2023	Tissue	Culture mycology	N	
Embekweni hospital	8/3/2023	Blood culture	Culture mycology	N	
Embekweni hospital	8/3/2023	Blood culture	Culture mycology	N	

embekweni hospital	8/3/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	8/3/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	8/3/2023	Sputum	Culture mycology	Y	*Candida albicans*
Universitas hospital	8/3/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	8/3/2023	Skin tissue	Culture mycology	N	
Universitas hospital	8/3/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital	8/4/2023	Tissue	Culture mycology	N	
Universitas hospital	8/5/2023	Tissue	Culture mycology	N	
Universitas hospital	8/5/2023	Tissue	Culture mycology	N	
Universitas hospital	8/7/2023	Sputum	Culture mycology	N	
Universitas hospital	8/8/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital	8/11/2023	Tissue	Culture mycology	N	
Universitas hospital	8/14/2023	Tissue	Culture mycology	N	
Universitas hospital	8/14/2023	Tissue	Culture mycology	N	
Universitas hospital	8/15/2023	Tissue	Culture mycology	N	
Universitas hospital	8/16/2023	Tissue	Culture mycology	N	

Pelonomi hospital	8/18/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	8/22/2023	Tissue	Culture mycology	N	
Universitas hospital	8/23/2023	Skin tissue	Culture mycology	Y	
Universitas hospital	8/23/2023	CSF	Culture mycology	N	
Universitas hospital	8/24/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	8/26/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	8/26/2023	Tissue	Culture mycology	N	
Pelonomi hospital	8/26/2023	Tissue	Culture mycology	N	
Universitas hospital	8/28/2023	Sputum	Culture mycology	Y	*Aspergillus fumigatus*
Universitas hospital	8/28/2023	Sputum	Culture mycology	N	
Universitas hospital	8/28/2023	Blood culture	Culture mycology	N	
Universitas hospital	8/28/2023	Nails	Culture mycology	Y	*Candida* species
Embekweni hospital	8/28/2023	Urine	Culture mycology	N	
Universitas hospital	8/29/2023	Blood culture	Culture mycology	N	
Universitas hospital	8/30/2023	Bronchial washing	Culture mycology	N	
Universitas hospital	8/30/2023	Tissue	Culture mycology	N	

Universitas hospital annex	8/30/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	8/31/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	8/31/2023	Blood	Culture mycology	N	
Pelonomi hospital	8/31/2023	Fluid / aspirate	Culture mycology	N	
September 2023					
DR js moroka hospital	9/4/2023	CSF	Culture mycology	N	
Universitas hospital	9/4/2023	Nails	Culture mycology	Y	*Alternaria* species
Universitas hospital	9/6/2023	Tracheal aspirate	Culture mycology	N	
Universitas hospital	9/6/2023	Swab (superficial)	Culture mycology	N	
Universitas hospital	9/7/2023	Tissue	Culture mycology	Y	*Acremonium* species
Universitas hospital annex	9/7/2023	Corneal scraping	Culture mycology	N	
Universitas hospital	9/8/2023	Tissue	Culture mycology	N	
Universitas hospital annex	9/9/2023	Swab (superficial)	Culture mycology	N	
Universitas hospital annex	9/9/2023	Swab (superficial)	Culture mycology	N	
Pelonomi hospital	9/10/2023	Blood culture	Culture mycology	Y	*Candida pelliculosa*
Universitas hospital annex	9/12/2023	Fluid / aspirate	Culture mycology	N	

Universitas hospital	9/12/2023	Blood culture	Culture mycology	N	
Universitas hospital	9/13/2023	Blood culture	Culture mycology	N	
Mohau hospital	9/13/2023	Subcutaneous effusion	Culture mycology	N	
Universitas hospital	9/14/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	9/14/2023	Tissue	Culture mycology	N	
Universitas hospital	9/14/2023	Tissue	Culture mycology	N	
National district hospital	9/15/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	9/18/2023	Sputum	Culture mycology	N	
Universitas hospital	9/18/2023	Swab (superficial)	Culture mycology	N	
Pelonomi hospital	9/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	9/20/2023	Tissue	Culture mycology	N	
Universitas hospital	9/20/2023	Tissue	Culture mycology	N	
Universitas hospital	9/21/2023	Skin tissue	Culture mycology	N	
Universitas hospital	9/21/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	9/21/2023	Blood culture	Culture mycology	N	
Pelonomi hospital	9/23/2023	Tissue	Culture mycology	N	

Pelonomi hospital	9/25/2023	Stool	Culture mycology	Y	*Candida* species
3 military hospital	9/26/2023	Sputum	Culture mycology	N	
Universitas hospital	9/26/2023	Sputum	Culture mycology	Y	*Aspergillus fumigatus*
Universitas hospital	9/27/2023	Blood culture	Culture mycology	N	
3 military hospital	9/27/2023	Sputum	Culture mycology	N	
3 military hospital	9/27/2023	Nails	Culture mycology	N	
National district hospital	9/28/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	9/28/2023	Sputum	Culture mycology	N	
pelonomi hospital	9/30/2023	Tissue	Culture mycology	N	
October 2023					
National district hospital	10/1/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	10/2/2023	Tissue	Culture mycology	N	
Pelonomi hospital	10/4/2023	Tissue	Culture mycology	N	
Universitas hospital	10/4/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	10/4/2023	Tissue	Culture mycology	N	
Universitas hospital	10/4/2023	Tissue	Culture mycology	Y	*Penicillium* species

Universitas hospital	10/9/2023	Blood culture	Culture mycology	N	
National district hospital	10/9/2023	Sputum	Culture mycology	Y	*Candida* species
Universitas hospital	10/10/2023	Blood culture	Culture mycology	N	
National district hospital	10/10/2023	Sputum	Culture mycology	N	
Universitas hospital	10/11/2023	Fluid / aspirate	Culture mycology	Y	*Candida albicans*
Universitas hospital	10/11/2023	TisSUE	Culture mycology	N	
Universitas hospital	10/13/2023	Skin tissue	Culture mycology	N	
Universitas hospital	10/13/2023	Skin tissue	Culture mycology	N	
Botshabelo hospital	10/14/2023	CSF	Culture mycology	N	
Embekweni hospital	10/16/2023	CSF	Culture mycology	N	
Universitas hospital	10/18/2023	Fluid / aspirate	Culture mycology	Y	*Aspergillus flavus*
Universitas hospital	10/20/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	10/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	10/23/2023	Tissue	Culture mycology	N	
Universitas hospital	10/24/2023	Tissue	Culture mycology	N	
Universitas hospital	10/24/2023	Tissue	Culture mycology	N	

Universitas hospital	10/24/2023	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	10/24/2023	Skin tissue	Culture mycology	N	
Pelonomi hospital	10/24/2023	Tissue	Culture mycology	N	
Pelonomi hospital	10/24/2023	Tissue	Culture mycology	N	
Phuthuloha hospital	10/25/2023	CSF	Culture mycology	N	
National district hospital	10/26/2023	Sputum	Culture mycology	Y	*Candida* species
National district hospital	10/26/2023	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	10/27/2023	Skin tissue	Culture mycology	Y	*Acremonium* species
Universitas hospital	10/27/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	10/27/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	10/28/2023	Tissue	Culture mycology	N	
Pelonomi hospital	10/28/2023	Tissue	Culture mycology	N	
Universitas hospital	10/31/2023	Blood culture	Culture mycology	N	
November 2023					
Universitas hospital	11/1/2023	Blood culture	Culture mycology	N	
Universitas hospital	11/1/2023	Blood culture	Culture mycology	N	

Universitas hospital	11/3/2023	Blood culture	Culture mycology	N	
Universitas hospital	11/3/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	11/6/2023	CSF	Culture mycology	N	
Pelonomi hospital	11/7/2023	CSF	Culture mycology	N	
Pelonomi hospital	11/7/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/7/2023	Tissue	Culture mycology	N	
Universitas hospital	11/8/2023	Bronchial washing	Culture mycology	N	
Universitas hospital	11/8/2023	Tissue	Culture mycology	N	
Universitas hospital	11/8/2023	Bronchial washing	Culture mycology	N	
Pelonomi hospital	11/9/2023	Tissue	Culture mycology	N	
Universitas hospital	11/10/2023	Sputum	Culture mycology	Y	*Candida* species
Phuthuloha hospital	11/10/2023	CSF	Culture mycology	N	
Pelonomi hospital	11/11/2023	Skin tissue	Culture mycology	N	
Pelonomi hospital	11/11/2023	Skin tissue	Culture mycology	N	
Universitas hospital	11/14/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	11/14/2023	Blood culture	Culture mycology	N	

Universitas hospital	11/15/2023	Bronchial alveolar lavage	Culture mycology	N	
Universitas hospital	11/15/2023	Bronchial washing	Culture mycology	N	
Universitas hospital	11/15/2023	Tissue	Culture mycology	N	
Universitas hospital	11/15/2023	Tissue	Culture mycology	N	
Embekweni hospital	11/16/2023	Tissue	Culture mycology	N	
Universitas hospital	11/16/2023	Tissue	Culture mycology	N	
Universitas hospital	11/16/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	11/19/2023	Tissue	Culture mycology	N	
Universitas hospital	11/19/2023	Tissue	Culture mycology	N	
Universitas hospital	11/20/2023	Fluid / aspirate	Culture mycology	N	
Universitas hospital	11/20/2023	Blood culture	Culture mycology	N	
Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	

Pelonomi hospital	11/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/21/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/21/2023	Tissue	Culture mycology	N	
Universitas hospital	11/25/2023	Blood culture	Culture mycology	N	
Universitas hospital	11/25/2023	Blood culture	Culture mycology	N	
Pelonomi hospital	11/27/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/27/2023	Tissue	Culture mycology	N	
Universitas hospital	11/28/2023	Blood culture	Culture mycology	N	
Universitas hospital	11/29/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/29/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/29/2023	Tissue	Culture mycology	N	
Pelonomi hospital	11/29/2023	Tissue	Culture mycology	N	
Universitas hospital	11/29/2023	Tissue	Culture mycology	N	
December 2023					
Pelonomi hospital	12/2/2023	CSF	Culture mycology	N	
Albert nzula district hospital	12/6/2023	Tissue	Culture mycology	N	

Albert nzula district hospital	12/6/2023	Tissue	Culture mycology	N	
Albert nzula district hospital	12/6/2023	Tissue	Culture mycology	N	
Albert nzula district hospital	12/6/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/6/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/6/2023	Tissue	Culture mycology	N	
Universitas hospital annex	12/7/2023	Corneal scraping	Culture mycology	Y	*Bipolaris* species
Pelonomi hospital	12/9/2023	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	12/9/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/11/2023	Tissue	Culture mycology	N	
Universitas hospital	12/12/2023	Tissue	Culture mycology	N	
Universitas hospital	12/12/2023	Fluid / aspirate	Culture mycology	N	
Embekweni hospital	12/12/2023	Sputum	Culture mycology	N	
Universitas hospital	12/13/2023	Tissue	Culture mycology	N	
Universitas hospital	12/13/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/13/2023	Tissue	Culture mycology	N	
Universitas hospital	12/13/2023	Skin tissue	Culture mycology	N	

Universitas hospital	12/13/2023	Skin tissue	Culture mycology	N	
Albert nzula district hospital	12/14/2023	CSF	Culture mycology	N	
Bongani regional hospital	12/15/2023	Sputum	Culture mycology	Y	*Aspergillus fumigatus*
Universitas hospital	12/17/2023	Tissue	Culture mycology	N	
Universitas hospital	12/17/2023	Tissue	Culture mycology	N	
Universitas hospital	12/18/2023	Tissue	Culture mycology	Y	*Alternaria* species
Universitas hospital	12/18/2023	Fluid / aspirate	Culture mycology	Y	MOULD
Universitas hospital	12/18/2023	Abscess (superficial) swab	Culture mycology	N	
Universitas hospital	12/18/2023	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	12/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/20/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/22/2023	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	12/22/2023	Blood culture	Culture mycology	N	
Pelonomi hospital	12/23/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/23/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/24/2023	Tissue	Culture mycology	N	

Pelonomi hospital	12/24/2023	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital annex	12/26/2023	Catheter urine	Culture mycology	Y	*Candida albicans*
Pelonomi hospital	12/28/2023	Tissue	Culture mycology	N	
Pelonomi hospital	12/29/2023	Tissue	Culture mycology	N	

January 2024					
Hospital	Date	Sample	Method	Growth	ID
Universitas hospital	1/4/2024	Tissue	Culture mycology	N	
Embekweni hospital	1/5/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/8/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/8/2024	Tissue	Culture mycology	Y	
Universitas hospital	1/8/2024	Fluid / aspirate	Culture mycology	N	
Embekweni hospital	1/9/2024	CSF	Culture mycology	N	
Pelonomi hospital	1/9/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/10/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/10/2024	Tissue	Culture mycology	N	
Universitas hospital	1/10/2024	Tissue	Culture mycology	N	

Universitas hospital	1/10/2024	Swab (superficial)	Culture mycology	N	
Universitas hospital	1/10/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/10/2024	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	1/10/2024	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	1/10/2024	Tissue	Culture mycology	N	
Universitas hospital	1/11/2024	Tissue	Culture mycology	N	
Universitas hospital	1/11/2024	Tissue	Culture mycology	N	
Universitas hospital annex	1/12/2024	Corneal scraping, culture plate inoculum	Culture mycology	N	
NHLS qa free state academic (ce)	1/16/2024	Tissue	Culture mycology	Y	*Aspergillus terreus*
NHLS qa free state academic (ce)	1/16/2024	Tissue	Culture mycology	Y	*Sporothrix schenckii*
NHLS qa free state academic (ce)	1/16/2024	Corneal tissue	Culture mycology	Y	*Trichophyton* species
Universitas hospital	1/17/2024	Tissue	Culture mycology	N	
Universitas hospital	1/17/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/17/2024	Tissue	Culture mycology	N	
Universitas hospital	1/17/2024	Tissue	Culture mycology	N	

Universitas hospital	1/17/2024	Tissue	Culture mycology	N	
Pelonomi hospital	1/19/2024	Blood culture	Culture mycology	N	
Universitas hospital	1/19/2024	Blood culture	Culture mycology	N	
Universitas hospital	1/23/2024	Fluid / aspirate	Culture mycology	N	
Universitas hospital	1/27/2024	Fluid / aspirate	Culture mycology	N	
Universitas hospital	1/29/2024	Tissue	Culture mycology	N	
Embekweni hospital	1/30/2024	Sputum	Culture mycology	N	
Universitas hospital	1/31/2024	Tissue	Culture mycology	N	
Universitas hospital	1/31/2024	Tissue	Culture mycology	N	
February 2024					
Pelonomi hospital	2/3/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/3/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/3/2024	Abscess (superficial) aspirate	Culture mycology	Y	
Pelonomi hospital	2/3/2024	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	2/4/2024	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	2/5/2024	Tissue	Culture mycology	Y	*Alternaria* species

Universitas hospital	2/5/2024	Tissue	Culture mycology	Y	*Epicoccum* species
Universitas hospital	2/5/2024	Tissue	Culture mycology	N	
Universitas hospital	2/5/2024	Tissue	Culture mycology	Y	*Epicoccum* species
Universitas hospital	2/5/2024	Tissue	Culture mycology	N	
Universitas hospital	2/5/2024	Tissue	Culture mycology	N	
Universitas hospital	2/5/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/5/2024	Tissue	Culture mycology	N	
Universitas hospital	2/7/2024	Tissue	Culture mycology	N	
Universitas hospital	2/7/2024	Tissue	Culture mycology	N	
Universitas hospital	2/7/2024	Blood culture	Culture mycology	N	
Universitas hospital	2/8/2024	Blood culture	Culture mycology	N	
Ganyesa community hospital	2/8/2024	Sputum	Culture mycology	N	
Universitas hospital	2/12/2024	Tissue	Culture mycology	N	
Universitas hospital	2/14/2024	Tissue	Culture mycology	N	
Universitas hospital	2/14/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/14/2024	Blood culture	Culture mycology	N	

Pelonomi hospital	2/14/2024	Blood culture	Culture mycology	N	
Pelonomi hospital	2/14/2024	Blood culture	Culture mycology	N	
Pelonomi hospital	2/14/2024	Skin tissue	Culture mycology	N	
Pelonomi hospital	2/17/2024	Skin tissue	Culture mycology	N	
Pelonomi hospital	2/20/2024	Sputum	Culture mycology	N	
Universitas hospital	2/21/2024	Bronchial alveolar lavage	Culture mycology	N	
Diamond hospital	2/21/2024	Fluid / aspirate	Culture mycology	N	
Pelonomi hospital	2/21/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/21/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/21/2024	Tissue	Culture mycology	N	
Pelonomi hospital	2/21/2024	Tissue	Culture mycology	N	
Universitas hospital	2/24/2024	Fluid / aspirate	Culture mycology	N	
Universitas hospital	2/26/2024	Skin tissue	Culture mycology	N	
Universitas hospital	2/26/2024	Abscess (superficial) aspirate	Culture mycology	N	
March 24					
Universitas hospital	3/1/2024	Tissue	Culture mycology	N	

Universitas hospital	3/1/2024	Tissue	Culture mycology	N	
Universitas hospital	3/1/2024	Blood culture	Culture mycology	N	
Universitas hospital	3/1/2024	Blood culture	Culture mycology	N	
Universitas hospital	3/1/2024	Blood culture	Culture mycology	N	
Universitas hospital	3/5/2024	Sputum	Culture mycology	N	
Universitas hospital	3/6/2024	Abscess (superficial) aspirate	Culture mycology	Y	*Alternaria* species
Universitas hospital	3/6/2024	Tissue	Culture mycology	N	
Universitas hospital	3/6/2024	Tissue	Culture mycology	Y	*Alternaria* species
Universitas hospital	3/6/2024	Fluid / aspirate	Culture mycology	N	
Universitas hospital	3/6/2024	Tissue	Culture mycology	N	
Universitas hospital	3/6/2024	Tissue	Culture mycology	N	
Universitas hospital	3/7/2024	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	3/7/2024	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	3/7/2024	Tissue	Culture mycology	Y	*Bipolaris* species
Universitas hospital	3/7/2024	Tissue	Culture mycology	Y	*Bipolaris* species
Universitas hospital	3/8/2024	Tissue	Culture mycology	N	

Universitas hospital	3/8/2024	Abscess (superficial) aspirate	Culture mycology	N	
Universitas hospital	3/8/2024	Tissue	Culture mycology	N	
Universitas hospital	3/8/2024	Abscess (superficial) aspirate	Culture mycology	N	
Pelonomi hospital	3/9/2024	Tissue	Culture mycology	N	
Pelonomi hospital	3/9/2024	Tissue	Culture mycology	N	
Universitas hospital	3/12/2024	Tissue	Culture mycology	N	
Universitas hospital	3/13/2024	Tissue	Culture mycology	N	
Universitas hospital	3/13/2024	Tissue	Culture mycology	N	
Universitas hospital	3/14/2024	Tissue	Culture mycology	Y	FUNGES
Universitas hospital	3/14/2024	Tissue	Culture mycology	N	
Universitas hospital	3/15/2024	Tissue	Culture mycology	N	
Universitas hospital	3/15/2024	Tissue	Culture mycology	N	
Pelonomi hospital	3/16/2024	Tissue	Culture mycology	N	
Universitas hospital	3/19/2024	Tissue	Culture mycology	N	
Phuthuloha hospital	3/19/2024	Swab (superficial)	Culture mycology	N	
Pelonomi hospital	3/21/2024	Intravenous catheter tip	Culture mycology	N	

Pelonomi hospital	3/22/2024	Tissue	Culture mycology	N	
Trompsburg municipal clinic	3/22/2024	Sputum	Culture mycology	N	
Universitas hospital	3/31/2024	Sputum	Culture mycology	N	
April 24					
Pelonomi hospital	4/1/2024	Sputum	Culture mycology	N	
Universitas hospital	4/3/2024	Sputum	Culture mycology	Y	*Cladodporium* species
Pelonomi hospital	4/3/2024	Tissue	Culture mycology	N	
John daniel newberry hospital	4/9/2024	Sputum	Culture mycology	N	
Pelonomi hospital	4/10/2024	Tissue	Culture mycology	N	
Universitas hospital	4/20/2024	Midstream urine	Culture mycology	Y	*Geotrichum* species

Chapter 4: Wastewater: A potential source of resistant yeast

(Parts of this chapter have been submitted for publication)

Risk for resistance selection towards fluconazole

Table S1 Measured fluconazole concentration for each site over 12 months in ng/L

	UFS	BV	WL	BW	SW	NNE	Total	Average
May	43.7	22.8	22.7	19.3	11.5	14.7	134.7	22.5
June	1.6	80.8	34.5	35.6	0	91	243.5	48.7
July	0.8	7.02	5.98	7.5	11.7	22.1	55.1	9.2
August	2.1	29.3	10.4	11.2	12.5	7.68	73.2	12.2
September	0.5	18.5	15.5	45.2	16.2	23.7	119.6	19.9
October	1.0	9.5	24.9	29.4	0	47.4	112.2	22.4
November	1.4	0	23.2	47.7	8.3	27.2	107.8	21.6
Desember	3.6	95.8	78.6	82.3	38	45.7	344.0	57.3
January	0	51.6	33.4	39.6	44.2	44.1	212.9	42.6
Febuary	0	10.4	33.7	40.4	37.8	41.3	163.6	32.7
March	0	31.2	37.7	42	95.8	52.3	259	51.8
April	0	6.25	2.7	4.0	1.7	5.94	20.7	4.1

Yeast fluconazole susceptibility testing

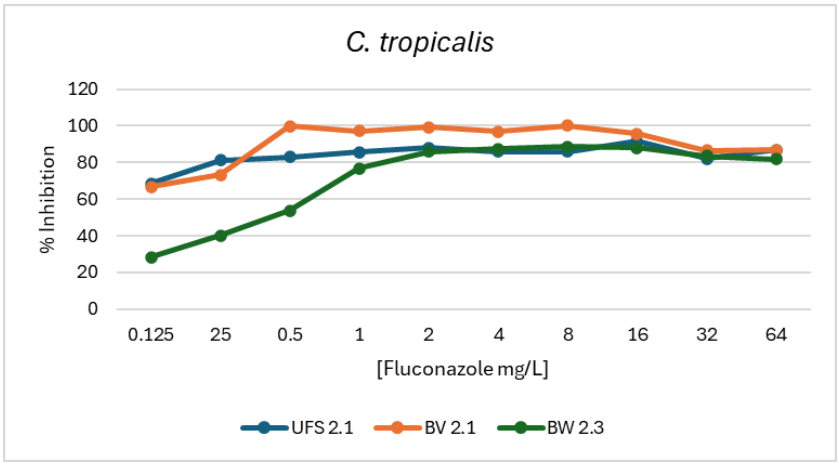


Figure S2A Percentage inhibition of *C. tropicalis*. Classified as susceptibility at fluconazole concentration ≤ 2 and resistant at concentrations of > 4 (EUCAST antifungal breakpoints)

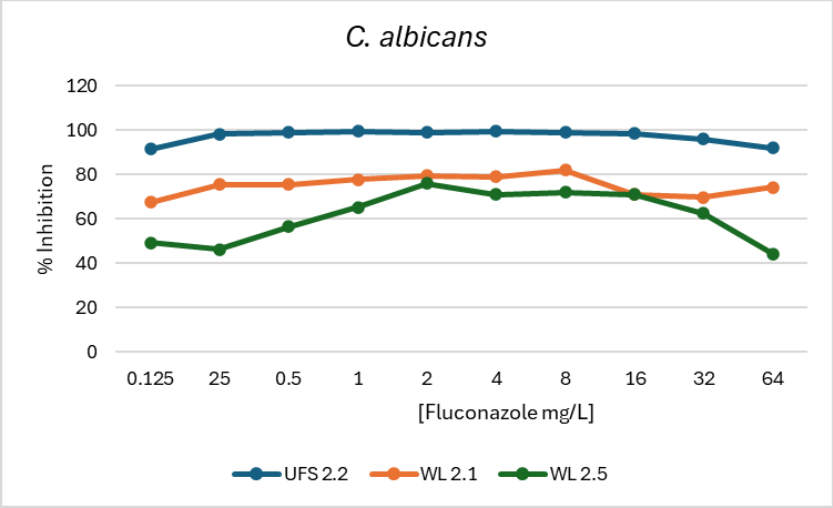


Figure S2B Percentage inhibition of *C. albicans*. Classified as susceptibility at fluconazole concentration ≤ 2 and resistant at concentrations of > 4 (EUCAST antifungal breakpoints)

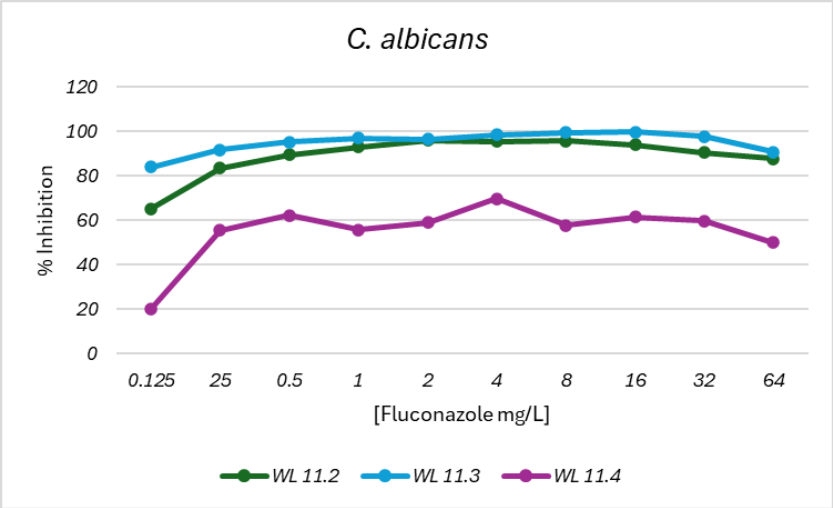


Figure S2C Percentage inhibition of *C. albicans*. Classified as susceptibility at fluconazole concentration ≤ 2 and resistant at concentrations of > 4 (EUCAST antifungal breakpoints)

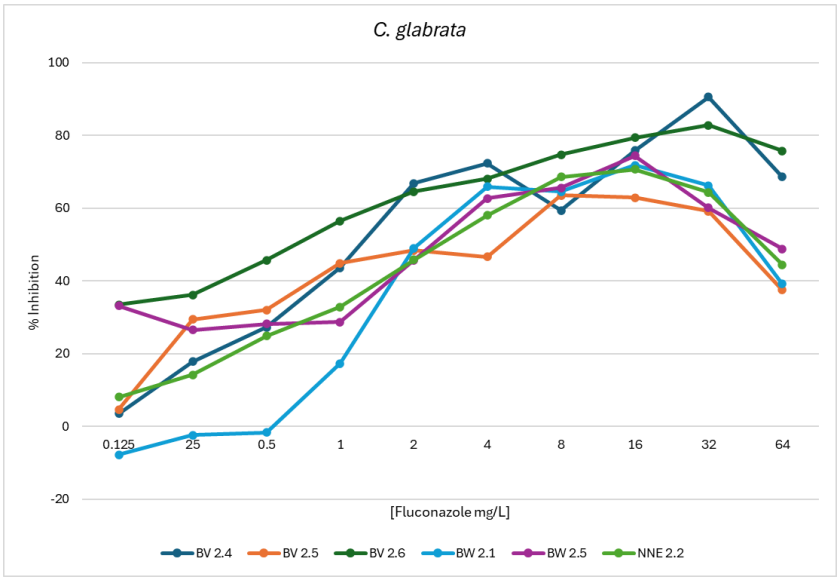


Figure S2D Percentage inhibition of *C. glabrata*. Classified as susceptibility at fluconazole concentration ≤ 0.002 and resistant at concentrations of > 32 (EUCAST antifungal breakpoints)

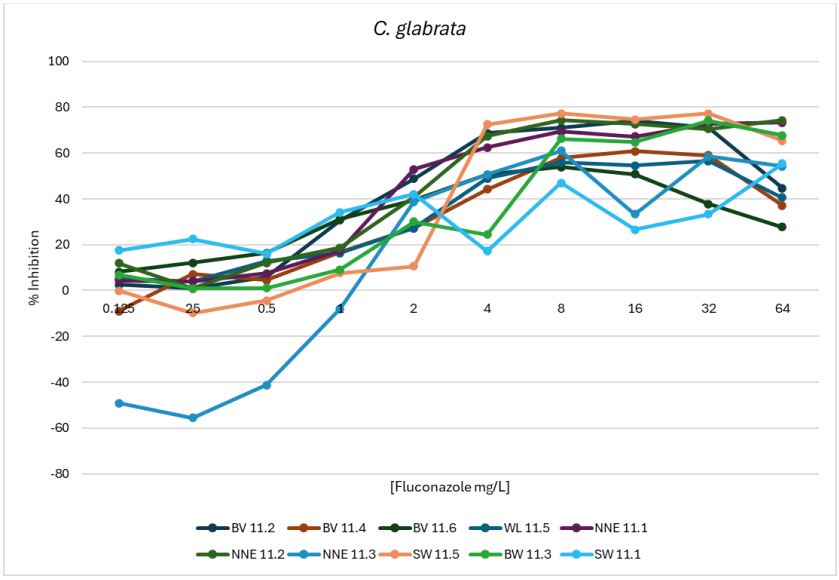


Figure S2E Percentage inhibition of *C. glabrata*. Classified as susceptibility at fluconazole concentration ≤ 0.002 and resistant at concentrations of > 32 (EUCAST antifungal breakpoints)

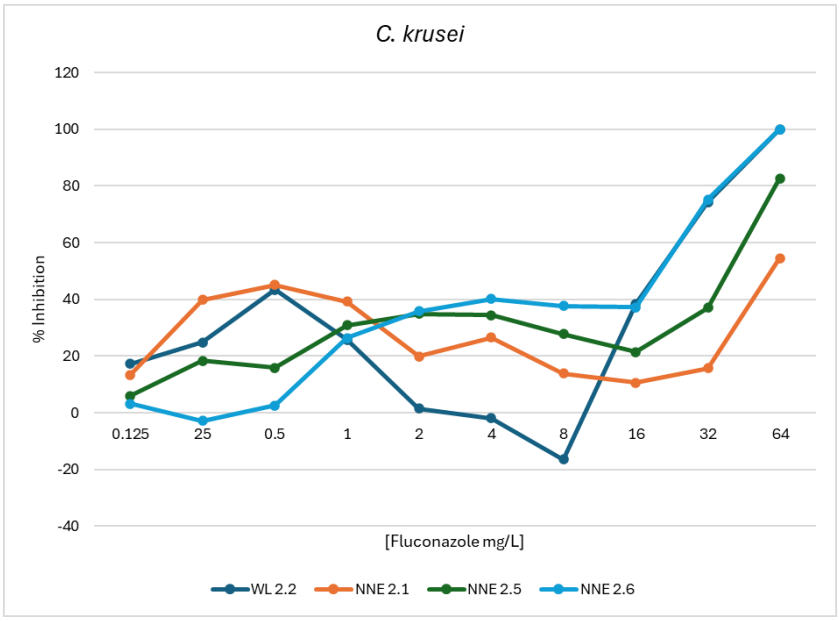


Figure S2F Percentage inhibition of *C. krusei*

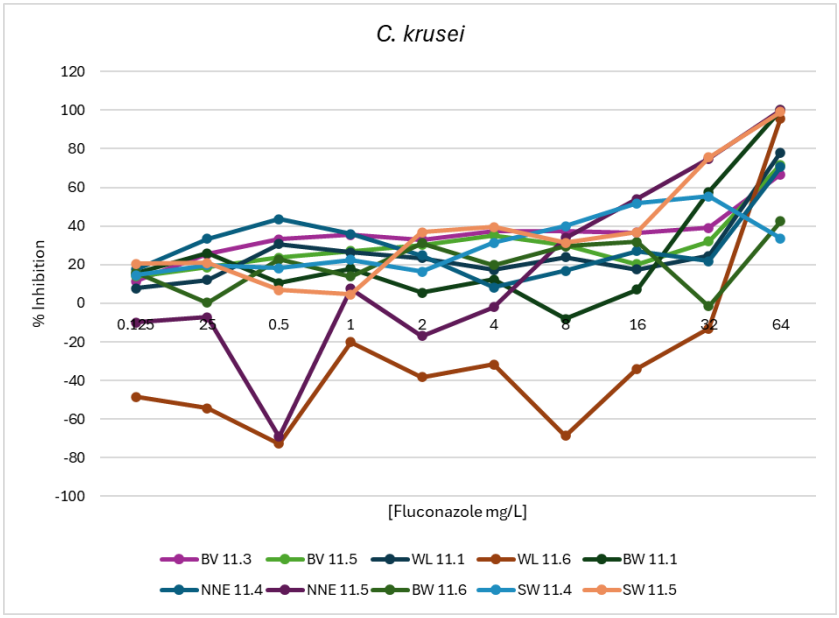


Figure S2G Percentage inhibition of *C. krusei*

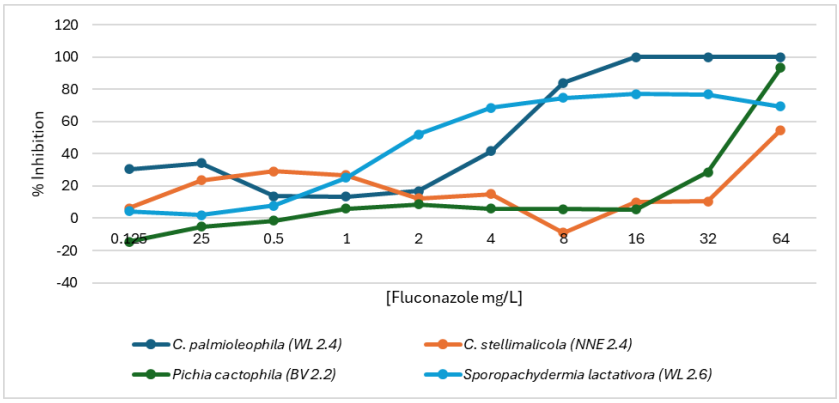


Figure S2H percentage inhibition of isolates. No EUCAST data reported

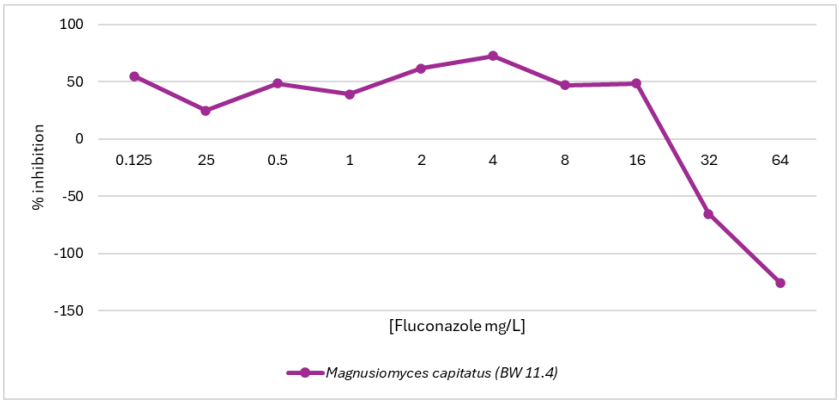


Figure S2I Percentage inhibition of *Magnusiomyces capitatus*. No EUCAST breakpoints reported

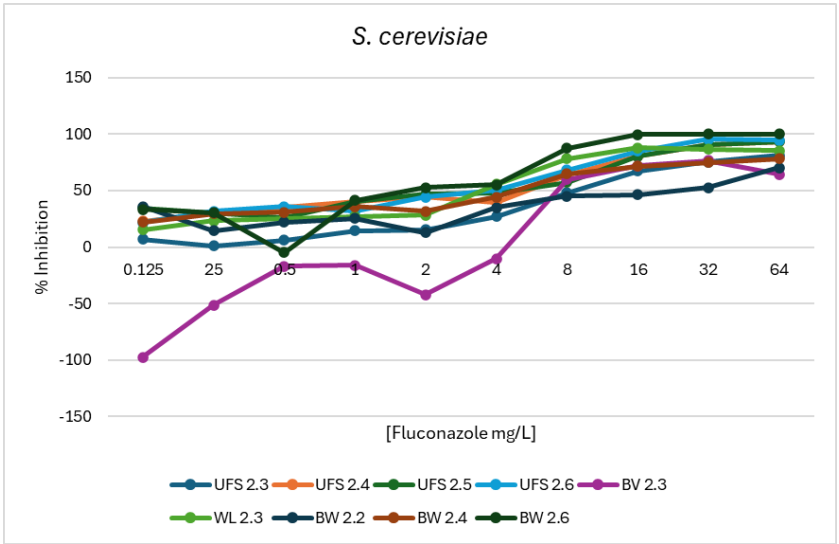


Figure S2J Percentage inhibition of *Saccharomyces cerevisiae.* No EUCAST breakpoints were reported for fluconazole

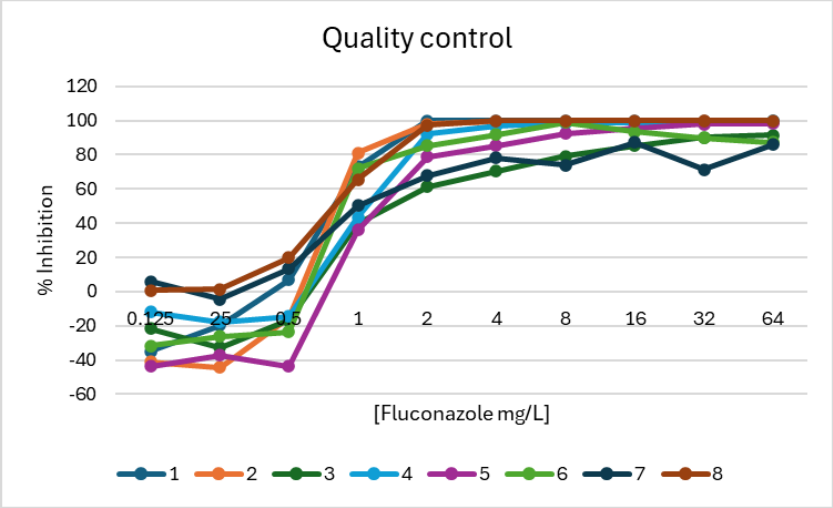


Figure S2K Percentage inhibition of *C. parapsilosis*. Classified as susceptibility at fluconazole concentration ≤ 2 and resistant at concentrations of > 4 (EUCAST Antifungal breakpoints)