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Riverine mycobiome dynamics: From South African tributaries to laboratory bioreactors

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ABSTRACT

Riverine fungi have the capacity for both pathogenicity, pertinent for countries with elevated immunosuppressed individuals, and bioremediation potential. The purpose was (i) to screen for the presence of clinically relevant riverine fungi and associations with anthropogenic influence, and (ii) the acclimatisation of environmental communities toward potential bioremediation application. Communities were harvested from polluted rivers in Stellenbosch, South Africa, and mycobiomes characterised by high-throughput amplicon sequencing. The remainder of the biomass was inoculated into continuous bioreactors with filtered river water or sterile minimal medium. Seven weeks later, the mycobiomes were re-sequenced. At least nine clinically relevant species were detected, including agents of mycoses belonging to the genus *Candida*. The occurrence of genera that harbour opportunisticstrains was significantly higher ($P = 0.04$) at more polluted sites. Moreover, positive correlations occured between some genera and pollution indices, demonstrating the potential of fungi for addition to water quality indicators. Despite biomass increase, almost all pathogens were undetectable after seven weeks, demonstrating less resilience in conditions mimicking rivers. Thus, when screening riverine biomes for bioremediation potential, ambient reactors select against human pathogens. This indicates a transient introduction of allochthonous opportunistic species into rivers due to insufficient sanitation, and the potential of bioremediation strategies that selects for environmental rather than pathogenic traits.

1. Introduction

Research on the presence and risks of microorganisms in surface waters, including rivers, is extensive; however, the focus is typically on bacteria and protozoa with a paucity of insight related to fungi (Chen et al. 2018; Yuan et al. 2019; Fregonesi et al. 2022). It is widely contended that climate change will result in the emergence of novel pathogens, with the most prominent of these likely to stem from the fungal domain (Steffen et al. 2023). These complex eukaryotes are highly adaptable to changing environmental conditions and are known to be tolerant to a range of adverse conditions, including elevated heavy metal concentrations, high osmotic pressures, and oligotrophic conditions (Mir-Tutusaus et al. 2018; Zhuo and Fan 2021). It is increasingly reported that fungi can adapt to grow at mammalian body temperatures and that clinically relevant fungal species are gaining resistance to the limited range of available antifungal agents (Lionakis et al.

2023). Moreover, there is strong evidence that increased pollution levels can be correlated to concurrent increases in clinically relevant fungal counts, particularly in polluted surface waters (Steffen et al. 2023). This group of microorganisms can pose a particular threat in countries such as South Africa (SA) with elevated numbers of immunocompromised individuals since opportunistic fungal infections are notoriously associated with immune burdens such as HIV and AIDS (Monapathi et al. 2020). In addition, inadequate sanitation infrastructure, serving informal settlements and poor urban areas, negatively impacts surface water quality and often results in the direct use of polluted water sources (Edokpayi et al. 2018).

South African water quality standards, although undergoing updates, include a limited selection of indicator organisms (bacteria, protozoa, and coliphages) which lack a fungal representative, despite the increasing number of local fungal infections in

combination with high numbers of immunosuppressed individuals (Assress et al. 2019). Similarly, global indicator lists contain no fungal representatives, although the inclusion of fungi may not be critical for all demographics. A primary reason for not including fungi in water quality testing may result from the paucity of fungal studies in polluted waters, with a concurrent lack of positive correlations observed with typical faecal indicators (Mhlongo et al. 2019). Clinically relevant fungal species are increasingly being detected in freshwater sources impacted by municipal sewage and industrial pollution (Chen et al. 2018; Assress et al. 2019; Samson et al. 2019; Monapathi et al. 2021). Moreover, due to their fundamental role in surface water ecosystems, fungi warrant inclusion as bioindicators of anthropogenic influence on freshwater bodies (Samson et al. 2019). Thus, a deeper understanding of the riverine mycobiome response to changing conditions is necessary to understand the dynamics of clinically relevant fungi in local water sources, and potential risks for water users. Such insight could also prove useful for the potential inclusion of fungal representatives in water quality monitoring if suitable characteristics were determined, to expand our current indicator organism suite.

Although fungi pose a human health burden, the aforementioned resilient qualities also render saprophytic fungi as particularly attractive bioremediation agents for polluted waters. Chemical and physical (i.e. ultrafiltration) processes are widely used and demonstrate success in the degradation of micropollutants (MPs); however, their limitations include increased waste generation, toxic by-products and high operational and financial demands (Sá et al. 2022). Therefore, biological treatments are gaining attention, with fungi gaining considerable interest in pollutant removal (Naghdi et al. 2018; Zhuo and Fan 2021). Zegzouti et al. (2020) observed the superior abilities of microorganisms autochthonous to contaminated sites for the degradation of complex pollutants. This is due to the ability to rapidly adapt and the efficient use of contaminants of interest within a reasonable period (David et al. 2018). Cultures showing promise for bioremediation are typically sourced from environmental locations that are polluted with the contaminant of interest, to harness environmental selection (Zegzouti et al. 2020). However, these sources are often contaminated with fungal pathogens too, particularly where faecal contamination is present (Assress et al. 2019; Steffen et al. 2023). Extra precaution

is advised when bioreactors are used to select for species with bioremediation capacity, from sources potentially rich in pathogens. Since pathogens relevant to humans are typically adapted to gut conditions (i.e. anaerobic, 37 °C, high organic load), it is presumed that natural selection towards bioremediation within laboratory bioreactors (cooler, more aerobic, lower organic load) will drive the community away from pathogen persistence. Therefore, an understanding of the mycobiome composition in environmental water sources can complement investigations of community dynamics in bioreactors, monitoring for the reduction of potential pathogens in addition to selecting promising bioremediators.

It has been demonstrated that correlations exist between the energy (i.e. ratios of nutrients carbon, nitrogen and phosphorus, COD, temperature) of an ecosystem and diversity, with evidence to support contrasting hypotheses (Qu et al. 2017; Ma et al. 2020). On the one hand, nutrient-rich environments could drive shifts towards less diverse communities, i.e. interspecies dominance of a few species and *vice versa* (Besemer 2015). However, when nutrients are scarce, species with improved degradation abilities invest energy to specialise, discouraging competition by creating an unfavourable environment via antagonism in the form of secondary metabolites and subsequently decreasing surrounding diversity (Drost et al. 2021). In terms of biodegradation, when a single species is compared to the efficacy of two or more species, diversity appears to facilitate degradation (David et al. 2018). However, when a sample with higher initial richness is compared to multi-species communities, a redundancy effect can occur when numerous species are able to break down the organic matter, hence diluting the importance of diversity for efficient biodegradation (Drost et al. 2021).

The aim of this study was to investigate dynamics from a less-studied fungal perspective within a mixed community setting, from their environmental niches to small-scale laboratory bioreactors. The riverine mycobiomes from urban rivers were investigated for their mycological pathogenicity and bioremediation potential. A broad range of micropollutants (MPs), widely acknowledged as a burgeoning threat to both surface and treated wastewaters (de Aquino et al. 2021; Szopińska et al. 2022), has been detected at the sampling locations used to source the river water medium for this study (Archer et al. 2017, 2023;

Holton et al. 2022). The samples were maintained in flow systems fed by either sterilised river water (containing several MPs) or a minimal medium (no MPs). Community shifts were expected to be driven by prior acclimatisation to pollution; thus, greater shifts were expected in samples from less pristine sites when adapting to increased pollution (MPs and physico-chemical parameters) in the river water used as reactor medium. Furthermore, natural environments are subject to constant flux that laboratory settings cannot mimic. Ecosystem health relies on a complex range of community interactions within which the role of fungi remains less explored; thus, the overall objectives were (1) to assess the survival of (mixed) fungal communities when moved from natural niches with continuous flux in nutrients and physical parameters into a rigid environment, maintained on natural and artificial media with fixed composition, and (2) to investigate the potential utility of autochthonous fungal communities in bioremediation, and towards the development of methods for early warning of persistence or potential proliferation of fungal pathogens.

2. Materials and methods

2.1. Sample collection

An overview of the study design is depicted in Figure S1. Environmental samples were collected from 10 locations (Table 1, Figure 1) along three river tributaries that flow through Stellenbosch (Western Cape, South Africa) representing relatively pristine conditions, in addition to sites subject to influence from respective urban and industrial activities.

Water samples (0.25–1 L) were collected in sterile Schott bottles midway between the surface and riverbed, after at least 7 days without rain, at the end of winter (August) with an average temperature range of 6–18 °C; the sampling was conducted at this time to provide a sequencing-based snapshot parallel to a year-long, culture-based study of yeast health risks in the same rivers (Steffen et al. 2023). Biofilm samples were scraped off the upper and undersides of the submerged rocks into sterile 50 mL Falcon tubes using sterile toothbrushes. All samples were transported on ice to the processing laboratory at the University of Stellenbosch, refrigerated (4 °C) within an hour of collection and were further processed on the same day of collection. Water parameters, including pH, conductivity and temperature, were measured on site using a multi-parameter instrument (PCTestr35™, Eutech, Paisley, UK), and dissolved oxygen (DO) was measured with a DO metre (ProODO; YSI, Yellow Springs, USA). Chemical oxygen demand (COD) and ammonium (NH_4^+) concentrations were calculated colorimetrically with Spectroquant technology (Supelco, Bellefonte, USA). Faecal contamination was assessed using *Escherichia coli* and coliform counts, using dilution series assessed on membrane lactose glucuronide agar (MLGA, Sigma-Aldrich, St Louis, USA) and incubating for 18 h at 37 °C.

2.2. Sample processing for high-throughput sequencing and inoculum preparation

The aqueous samples were aseptically filtered through 47 mm, 0.45 μm (pore size) GN-6 Metricel® filters (Pall Corporation, NY, USA). The turbidity of the samples was determined by the volume filtered per site, controlling for consistent biomass rather than volume, and ranged from 0.35 L (Site 1) to 2 L (Site 6). The filters were transferred to petri dishes containing 4 mL of 0.3 mol/L citrate buffer (pH 3.5) (Waso et al. 2016). After 3 min, solid matter was scraped off with a sterile blade and the filters were discarded. The aqueous suspensions were consecutively and cumulatively centrifuged in 2 mL microcentrifuge tubes

Table 1. The locations and descriptions of river samples evaluated in Stellenbosch, Western Cape, South Africa.

Site		Tributary	Notes	Samples
HP (highly polluted)*	1	Plankenbrug	Downstream of informal settlements/adjacent to industry	Water, biofilm
	2	Plankenbrug	After Krom confluence	Water
	3	Krom	Next to low-income housing/subject to pollution/direct use	Water
	4	Krom	Further human/industry impact	Water
	5	Eerste	After Plankenbrug confluence	Water, biofilm
LP (less polluted)*	6	Eerste	Minimal human impact after catchment in a nature reserve	Water, biofilm
	7	Eerste	Limited impact from town outskirts	Water, biofilm
	8	Eerste	Downstream of 7 before confluence with Plankenbrug	Water
	9	Veldwagters	WWTP effluent 0.8 km (primarily farmland) from discharge	Water
	10	Eerste	Downstream from all the above	Water

*Grouping based on qualitative observations (in table) and quantitative measurements including *Escherichia coli*, coliforms, dissolved oxygen (DO), and chemical oxygen demand (COD).
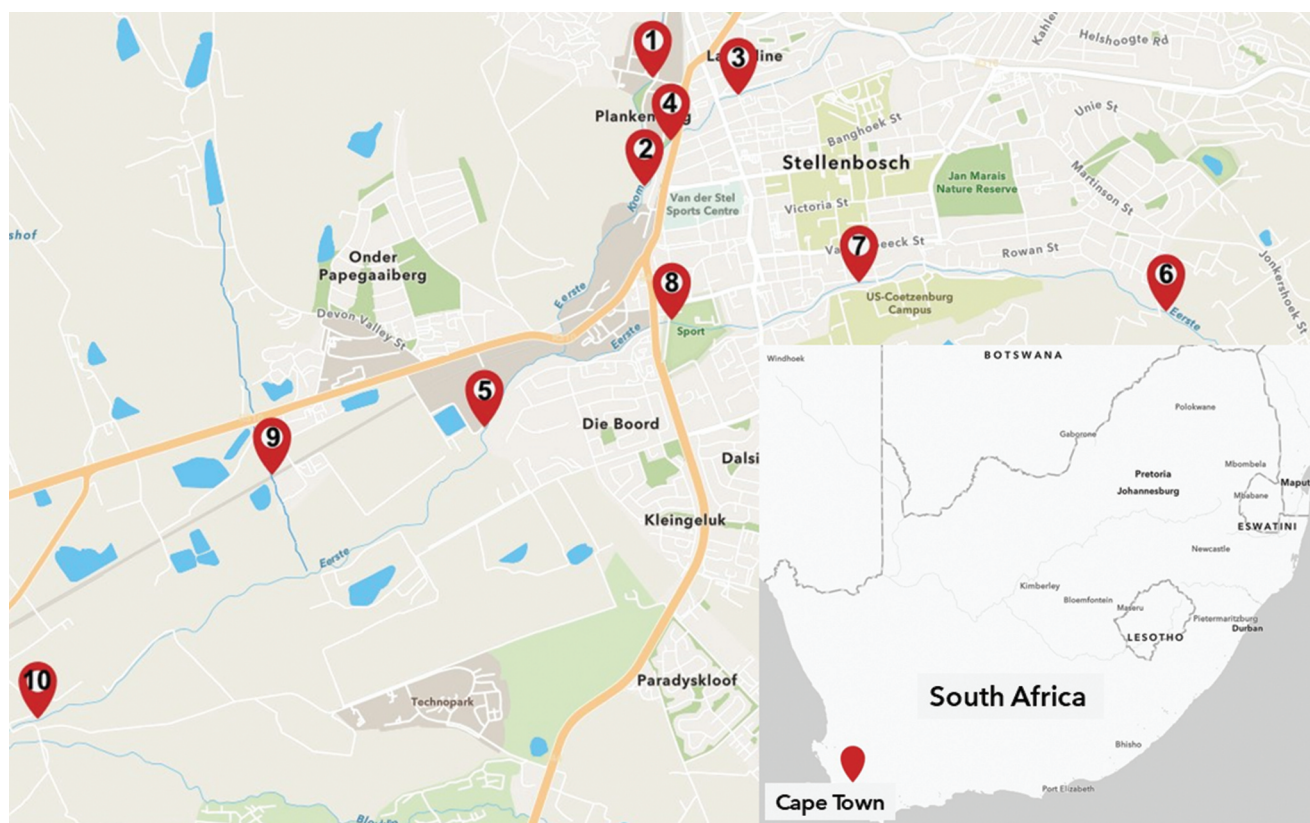
Figure 1. Riverine sampling locations. Site details provided in Table 1.

(15,000 × g, 2 min) and the supernatant was discarded. The biofilm samples were centrifuged for 15 min at 5,000 × g, the supernatant was discarded, and 300 mg of the pellet was used per sample. In both cases, the pellet was resuspended in 200 µL phosphate-buffered saline (PBS).

This preparation was performed in duplicate per sample site and type (10 aqueous and 4 biofilm samples; $n = 14$), with triplicates acquired from two sites for untreated ethidium monoazide (EMA) controls to compare community data for quality control. The first set of samples was used for DNA extractions, preceded by an EMA treatment (Section 2.4). The second set of aqueous samples was spun down a final time, the remaining citrate buffer discarded, and these concentrated samples were consolidated with the biofilm samples to inoculate bioreactors for evaluating acclimatisation.

2.3. Acclimatisation to environmental levels of organic pollutants

Figure S2 provides an overview of the acclimatisation setup to assess the survival of environmental communities in filtered river water, containing a broad range of MPs at measured environmental concentrations (Holton et al. 2022; Archer et al. 2023), as a test medium and a minimal medium as control. Twelve glass bioreactors were sterilised before adding 100 biofilm carriers to each (9 × 14 mm, surface area 1,200 mm^2/carrier; Ecotao Enterprises, Cape Town, South Africa) and 300 mL starting media. Six reactors received M63 minimal medium with 2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 0.5 mg $FeSO_4 \cdot 7 H_2O$, 1 mL $MgSO_4 \cdot 7 H_2O$ solution (1 mol/L), 0.1 mL Vitamin B_1 (thiamine) solution (0.5%) and 5 mL CasAmino acid solution (20%). This serves as a control medium with the absence of micropollutants (MPs). The other six reactors received river water collected weekly from Sites 1 and 9, combined in equal ratios (6.5 L per site/week); this was designed to represent a wider range of MPs, in addition to introducing weekly variations to the bioreactors that more accurately represent the natural flux expected in a river system. Degradation of MPs in experimental setups can occur due to photolysis when photosensitisers in the water matrix promote the generation of reactive species (Mathon et al. 2021;

Guo et al. 2022). Thus, to achieve a more consistent MP exposure, the river water medium was replaced each week. Previous research by our group showed a broad selection of MPs at these two sites (Holton et al. 2022). In order to acclimatise the inocula to a complex mixture of MPs, the consolidated river water medium required sterilisation without degradation of the MPs. To this effect, the water was passed through a series of filters with a final aseptic filtration under vacuum using sterile cellulose nitrate filters (47 mm, 0.2 μm pore size; CHMLAB Group, BCN, Spain), removing only microbes and particulate matter.

Chemical parameters were continuously measured for the weekly river water influent and once-off for the minimal medium. Selected parameters were also assessed for the effluent of the bioreactors from a single collection before the experiment was terminated. Total organic carbon (TOC) was tested using an Elementar Cario TOC cube (Elementar, Germany) and COD, total phosphorus (TP) and total nitrogen (TN) were measured and calculated using the respective Spectroquant® Cell Tests (Merck Millipore).

The consolidated inocula for the bioreactors were based on two groupings; namely, Sites 1–5 and 6–10, with the former categorised as highly polluted (HP), and the latter less polluted (LP). This grouping was based on qualitative observations (Table 1), and quantitative measurements, including *E. coli*, coliforms, DO, and COD. To prepare the consolidated HP and LP inocula, the respective aqueous (five sites) and biofilm (two sites) biomasses were combined in a 50 mL Falcon with 21 mL PBS, for each grouping. From the biofilm pellets, 1.2 g from each of the two site samples was used per grouping. From the two consolidated inocula, 3 mL was removed and centrifuged. The supernatant was discarded, and the pellet was resuspended in 200 μL PBS for EMA treatment and DNA extractions (Section 2.4). Six sterile bioreactors were each aseptically inoculated with 3 mL of the remaining 18 mL inoculum, for the HP and LP groups, respectively (n = 12).

The 12 bioreactors were maintained for 7 weeks (49 d), allowing enough time for fluctuation and re-stabilisation of communities (Artigas et al. 2012; Rodarte-Morales et al. 2012; Mir-Tutusaus et al. 2017) at ±24 °C (a temperature that supports the growth of most fungi) under continuous flow with a hydraulic residence time of 25 h (flow rate 12 mL/h) controlled by a 12-channel peristaltic pump (Figure S2), and

manually swirled twice a week. The medium was pumped through silicone tubing (1.6 mm inner/3 mm outer diameter). Silicone sealant was used to seal the entrance of the tubing through the lids into the glass jars and the exit of the overflow tubing. Exit holes were drilled beforehand, and the inserted overflow tubing had a 35 mm outer diameter.

The bioreactors were sampled after 49 d. The biomass was loosely attached to the carriers; thus, the reactors were vigorously shaken for 1 min by hand to detach the biomass for representative biofilm biomass. The carriers were removed, and the remaining media were cumulatively centrifuged in autoclaved containers at 2,500 × g until all biomass was collected per reactor (n = 12). A 300 mg portion of the pellet was separated and resuspended in PBS for EMA treatment and DNA extractions (Section 2.4), and the remainder was stored in glycerol at −80 °C. It should be noted that no bacterial inhibitors were included in this experiment. Thus, although the fungal counterparts were targeted for analysis, these species dynamics developed within the more realistic setting of mixed communities.

2.4. EMA treatment and DNA extractions

The concentrated river (1 mL), biofilm (300 mg) and bioreactor pellet (300 mg) samples were treated with 2.5 μg/mL EMA (Reyneke et al. 2017). After EMA addition, samples in 2 mL Eppendorf tubes were vortexed and incubated on ice in the dark for 10 min, followed by 15 min under halogen light for 10 min with the samples horizontally on ice at a distance of 20 cm. The samples were then washed with 1 mL NaCl (0.85%), centrifuged (16,000 × g, 5 min) and the supernatant discarded before resuspending in 200 μL PBS. Two samples were used as untreated controls (the third replicates were collected from two sites as mentioned in Section 2.2).

Total DNA was extracted using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA). The resuspended pellet was transferred to ZR BashingBead™ lysis tubes whereafter manufacturer's instructions were followed. Briefly, the binding preparation with the addition of ethanol was used for all samples, an additional centrifugation step was added after the second wash buffer (2) step (to clear

excess liquid from the IICR column) and DNA was eluted with 60 µL DNase/RNase Free Water. The DNA samples were stored at 4 °C until further analysis.

2.5. PacBio sequencing and data analyses

All DNA samples were sent for PacBio high-throughput amplicon sequencing at Inqaba Biotec (Pretoria, South Africa). The analysis of ITS gene amplicons was performed on the Sequel system by PacBio (www.pacb.com), using 5'-CTTGGTCATTTA GAGGAAGTAA-3' (ITS1F) and 5'-TCCTCCGCTTA TTGATATGC-3' (ITS4) primers. Raw subreads were processed through the SMRTlink (v8.0.0.80529) Circular Consensus Sequences (CCS) algorithm to produce highly accurate reads (>QV40).

2.5.1. Sequence processing

The raw sequence data were submitted to GenBank as Fastq files with accession number SAMN35537063. The raw sequence data were analysed using Mothur (v.1.44.1), with some modifications for the fungal ITS region (Schloss et al. 2009). In short, sequences that were between 100 and 1,000 bps in length, with an average quality score of 25 and higher, no ambiguous bases, and containing homopolymer regions shorter than 8 base pairs, were selected for further analysis. Chimeric sequences and sequences with errors were removed to ensure that only high-quality sequences were classified using the latest available UNITE reference database. Sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity and classified using a cut-off value of 80%. All samples were normalised to contain the same number of sequences.

2.5.2. Statistical analysis

All statistical analyses were performed in Mothur (v.1.44.1) and R (v.4.1.0, R Core Team 2021) using the microeco package (Liu et al. 2021). Non-parametric Kruskal-Wallis H-tests were calculated for all alpha diversity metrics. Multidimensional scaling plots (PCoA) were drawn in R using the Bray-Curtis dissimilarity matrix. Further statistical evaluations of the PCoA plots were conducted using Permutational Multivariate Analysis of Variance (PERMANOVA). Spearman correlation tests were performed between environmental measures and PCoA ordinations based on the Bray-Curtis dissimilarity matrix. Distance-based redundancy analysis (RDA) and heatmaps were used to further explain the variation observed in beta diversity. Differences in clinically relevant fungal abundance between pollution groups were assessed using the Mann-Whitney test. For all statistical evaluations, a P-value below 0.05 was considered significant.

3. Results and discussion

3.1. Water quality parameters

The results for the faecal water quality indicators (Figure S3) at the 10 riverine locations illustrate the motivation for the grouping of Sites 1–5 and 6–10, respectively, for the acclimatisation experiment.

3.2. Opportunistic or potentially pathogenic fungi

In total, 20 genera that may include opportunistic or pathogenic agents (Köhler et al. 2015; Assress et al. 2019; Aliyu et al. 2020; Kumari et al. 2021; Steffen et al. 2023) were detected across 14 samples from 10 riverine locations (Figure 2), with an overall average abundance, relative to the total fungal population in the sample, of 0.97%. The limitations of an isolated sampling event, in addition to the lack of selective culturing, should be noted, i.e. the possibility of higher pathogen risk in the environment than observed in this study. Moreover, the occurrence of non-pathogenic species from the genera *Cladosporium*, *Penicillium*, *Pichia*, and *Saccharomyces* in the environment or human mycobiota should be acknowledged (Magwaza et al. 2017; Assress et al. 2019; Lionakis et al. 2023). Nevertheless, as predicted, the average abundance of human-associated genera overall was at least threefold higher at the HP sites than at the LP sites ($P = 0.04$). Moreover, the abundances of eight genera containing opportunistic agents, as calculated for Site 1 only (from the HP group), were significantly higher (eightfold) than the average abundances of these genera across the LP group ($P < 0.01$) (Figure 3). Site 1 was the most polluted based on quantitative (Table 1) and qualitative assessment (proximity to informal settlement discharge and unsanitary human activity).

Many severe fungal infections in humans are caused by representatives of the genera *Aspergillus*, *Candida*, and *Cryptococcus* (Boral et al. 2018; Kaki 2023). However, considering that both *Aspergillus*
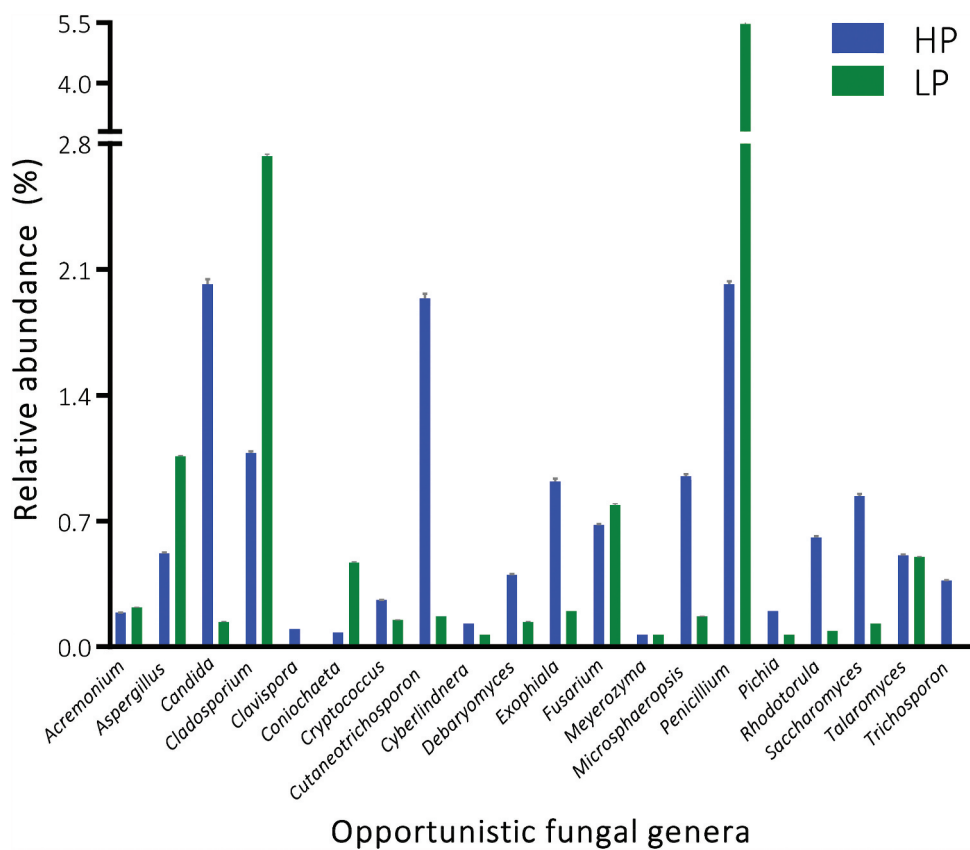
Figure 2. Relative abundance, as average of seven samples (5 × water and 2 × biofilm from five locations), of fungal genera harbouring opportunistic and pathogenic species detected in rivers. LP: Less polluted; HP: Highly polluted.
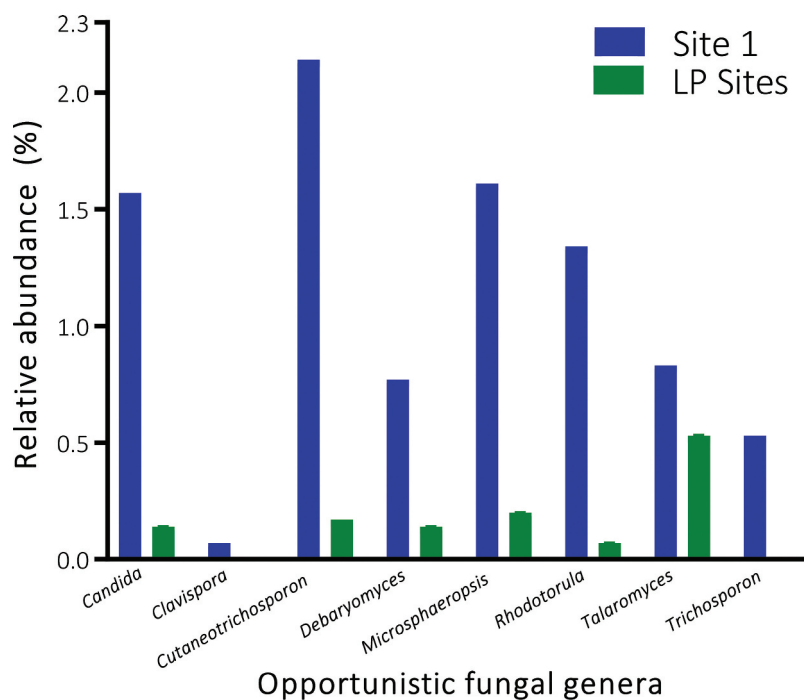
Figure 3. Average relative abundance of select genera in riverine samples from the Stellenbosch area. Site 1: Highly polluted; LP: Less polluted (average relative abundance across five locations).

and *Cryptococcus* also contain numerous non-pathogenic species abundant in environmental settings (Casadevall et al. 2017), it is unsurprising that their occurrence was not restricted to highly polluted sites, although *Cryptococcus* was more abundant at HP sites. Furthermore, three of the most prevalent causes of fungal infections in humans, *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis*, were detected only at Site 1, with *Candida parapsilosis* present in higher abundance in the biofilm sample. Considering the higher infection rate of immunosuppressed individuals by these species, it is cause for concern due to the proximity of human settlements to Site 1 (Casadevall et al. 2017).

Clavispora lusitaniae and an unclassified *Trichosporon* sp. were both detected only at Site 1 and one other HP site. The genus *Trichosporon* contains 50 species, of which 16 have been implicated in mycoses among immunosuppressed patients that range from superficial to severe or even fatal cases (Hospenthal 2015; Li et al. 2020). Thus, the unclassified sp. observed at Site 1 could be of human origin, considering the absence of environmental *Trichosporon* strains at this and all other sites.

The opportunistic pathogen *C. lusitaniae* has been implicated in up to 2% of candidemia cases in clinical settings (Desnos-Ollivier et al. 2011). The dematiaceous fungus, *Microsphaeropsis arundinis*, was also found to be one of the dominant clinically relevant fungi at Site 1 (Figure 3). This fungus is increasingly being reported to infect soft tissues in immunocompromised patients (Reppas et al. 2015).

Representatives of *Rhodotorula* are ubiquitous in nature, and three of these are increasingly responsible for disease in susceptible individuals, with *Rhodotorula mucilaginosa* being the most prevalent of the three to cause fungemia (Castón-Osorio et al. 2008; Wirth and Goldani 2012). Both *M. arundinis* and *R. mucilaginosa* displayed their highest relative abundances at Site 1, with detection in most HP samples, yet limited presence at LP sites. The *Talaromyces* genus also contains several clinically relevant species reported to cause mild to fatal infections, including *Talaromyces marneffei,* which is primarily associated with AIDS-affected individuals (Sun et al. 2020). *Cutaneotrichosporon* and *Exophiala* (Figures 2 and 3) were a further two genera much more abundant in the HP group, with three clinically relevant *Exophiala* species detected of which two (*Exophiala oligosperma*

and *Exophiala xenobiotica* detected at Site 3) are associated with systemic infection (Zeng et al. 2007).

In addition to the pathogens themselves, an acknowledged health risk due to fungal presence in surface waters is the associated mycotoxins that are being increasingly incorporated into water quality assessments (Magwaza et al. 2017; Mhlongo et al. 2019). Over half of the genera with representatives known to produce toxins were observed in this study, namely *Acremonium, Aspergillus*, *Exophiala*, *Fusarium*, *Cladosporium*, and *Penicillium*, the latter two of which were the most dominant opportunistic fungal genera across all sites.

The relative abundance of several fungal genera was strongly correlated with the faecal pollution indicators *E. coli* and coliforms (Figure 4). Monapathi et al. (2021) also observed significant associations between yeast numbers and faecal coliforms in two northern South African rivers, measured using qPCR and 26S sequence analysis. Overall, parameters such as temperature, ammonia, pH, and DO demonstrated less effect on the abundance of these genera although COD levels, an indicator of organic pollutants, showed positive correlations with the numbers of *Paramicrosporidium*, *Tausonia*, and a genus of the phylum Rozellomycota.

A review by Mhlongo et al. (2019) reported a general lack of conclusive correlations between faecal indicators and fungi in treated drinking water systems. Similarly, relatively low faecal indicators were observed in the (diluted) wastewater treatment plant (WWTP) effluent (Site "9", Figure S3) as expected after disinfection processes; however, eight of the opportunistic fungal genera (Figure 2) were still detectable in relative abundances of 0.1%–4.6% (data not shown), supporting possible resilience to current disinfection practices. The limited correlations between yeast and faecal indicators observed thus far may be due to fungi demonstrating improved resistance towards disinfection compared to bacteria (Mhlongo et al. 2019), which alarmingly may be the case for some treated water. While this resilience alone is the motivation for their inclusion in water monitoring practices, 11 genera in this study were positively associated with both *E. coli* and coliforms (Figure 4) and five of these are also associated with the human gut, namely *Candida, Cutaneotrichosporon*, *Geotrichum*, *Rhodotorula*, and *Saccharomyces* (Hallen-Adams and Suhr 2017; Nash et al. 2017). Moreover, in relation to indicator
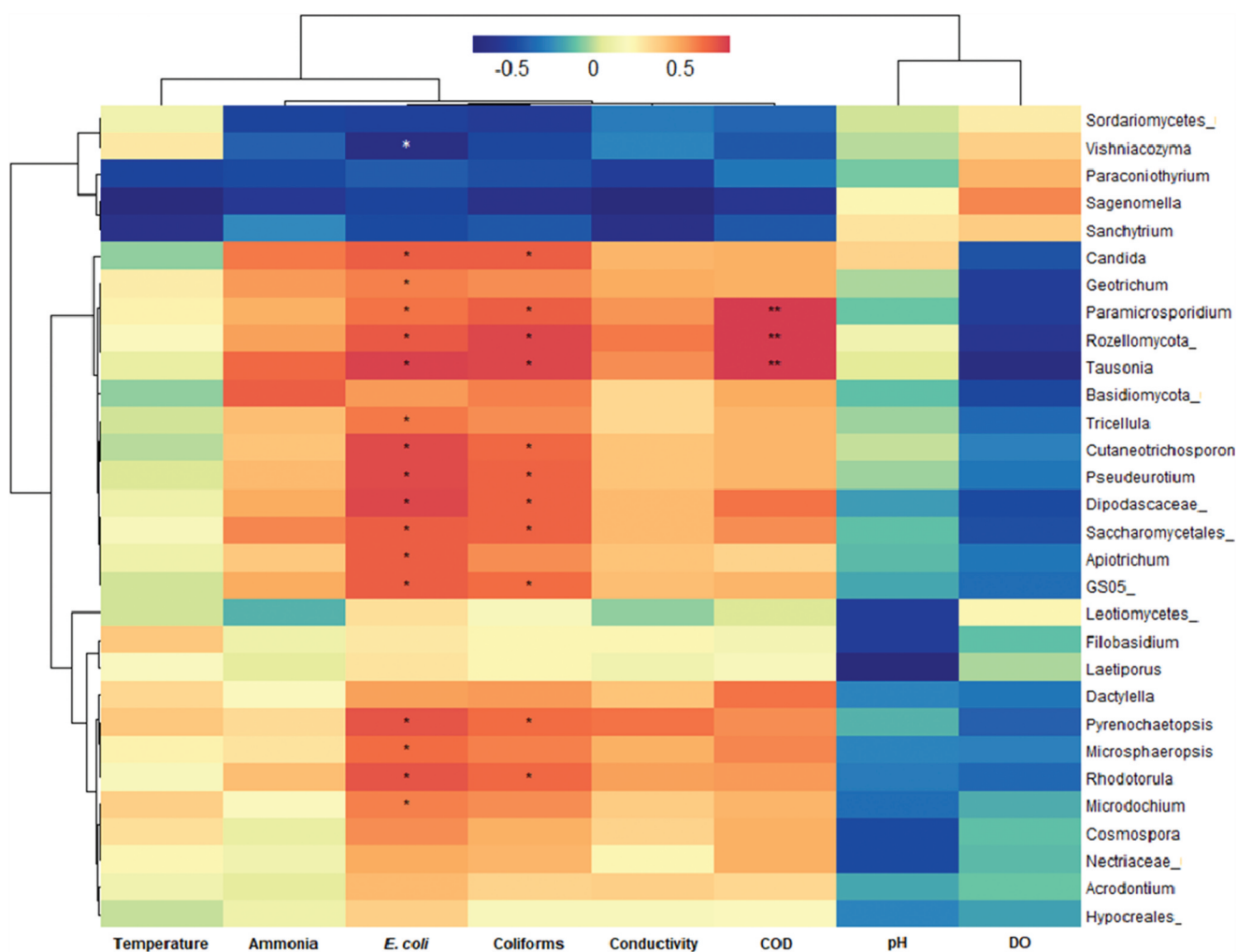
Figure 4. Hierarchically clustered heatmap of the 30 genera from river water communities across 10 sample sites most influenced by tested water quality parameters. */**: 0.01/0.001 significant correlation (positive/negative). Classification followed by "_": Unclassified genus (preceded by highest taxonomic identification).

requirements, the five above genera linked to the human intestinal tract have also been detected in a variety of water samples, demonstrating resilience in aqueous environments. Notably, representatives of these genera are quantifiable by simple and inexpensive culture methods, combined with an incubation temperature of 37 °C to select for opportunistic fungi (Babič et al. 2017; Chen et al. 2018; Sautour et al. 2021). *Candida albicans*, detected at the most polluted site (Site 1) and not further downstream, is more commonly associated with the mammalian gut than the natural environment (Köhler et al. 2015; Sautour et al. 2021). Furthermore, although some levels of *Candida* were detected in most of the bioreactors after 7 weeks, clinically relevant species were not among these, supporting the likelihood of a transient and human-associated presence in environmental waters. Thus,

there is motivation to further explore the potential utility of these fungal genera as suitable indicator species.

Regarding the observed decrease in pathogen abundance, as predicted for the acclimatisation experiment and mentioned above for *Candida*, none of the 13 potentially pathogenic fungal species observed before the acclimatisation period was detected with sequencing methods after 7 weeks in the bioreactors. Of the 20 pathogen-inclusive genera, only 10 remained after 7 weeks, and the relative abundances of the latter were all lower in the final reactor communities than in the initial inocula with the exception of *Rhodotorula*. Pathogenic *Rhodotorula* species are known to occur in a range of habitats including tap water, indicating resilience or ability to persist in oligotrophic conditions resulting from the inhabitation of diverse niches (Wirth

and Goldani 2012; Babič et al. 2017). The decrease in the number of potential pathogens (data not shown) is likely due to environmental conditions driving community shifts away from the typical mammalian host niche, such as a lack of elevated temperature (37 °C). The bioreactors were at ±24 °C thus evolutionary pressure may have favoured the environmental species over the opportunistic agents. Moreover, high nutrient-loading and anaerobic areas within the varied habitats of the rivers can promote niches that better represent the human body. Thus, in the contrasting bioreactor environment, they may have succumbed to competition from better adapted oligotrophs, particularly species autochthonous to the river. This result points to the potential of utilising acclimatised environmental samples as inoculum for environmental restoration, where evolutionary pressure away from the enrichment of opportunistic pathogens reduces the risk for personnel of exposure to pathogens.

3.3. Fungal community composition

3.3.1. Environmental: water and biofilm matrices
The respective fungal community compositions of the HP and LP groups were significantly different from each other ($P = 0.001$). Site 10 represents a confluence of all the other sites; thus, the positioning at the overlap between the two groupings (Figure 5) reflects the community composition expected from this sampling niche and results in less association with the LP group alone. Site 8, less aligned with either group, may reflect the close proximity to unique urban influences including surrounding schools, small businesses, and residential and experimental farm activities. Site 9 (primarily WWTP effluent) was also disassociated from both groupings, likely due to the upstream treatment processes altering the natural fungal communities and causing the observed shift away from the other communities (Figure 5). The presence of several genera known to contain opportunistic pathogens at this location may indicate the inefficient removal of fungi within the WWTP and resilience of certain species to treatment protocols, or even the potential selection towards antimicrobial resistance within the WWTP, an increasingly reported phenomenon globally (Frascaroli et al. 2021).

Some river biofilm samples were included in this study to compare planktonic and sessile community profiles from the same locations. The different matrix
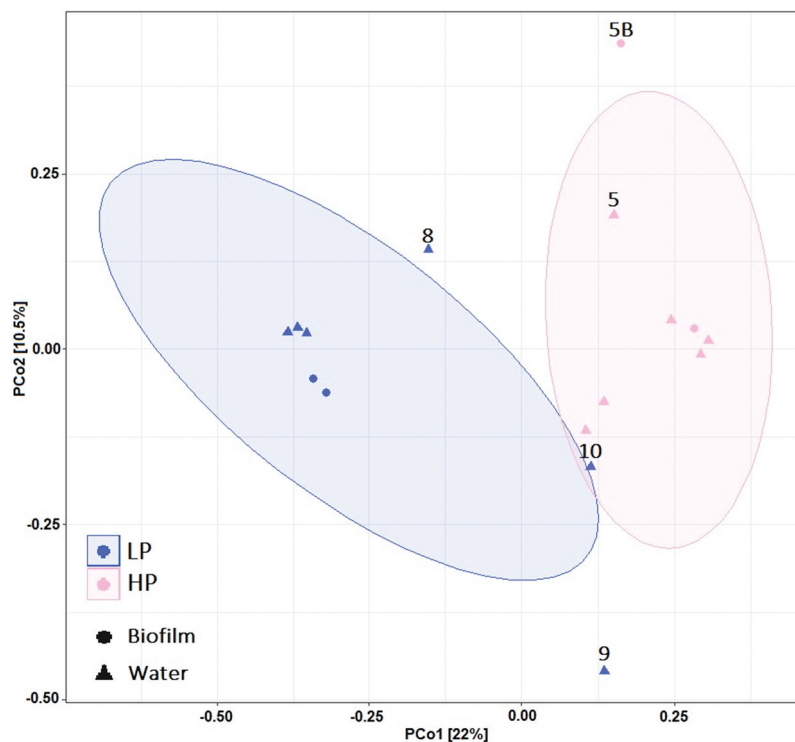
Figure 5. Principal coordinate analysis plots (PCoA) of fungal communities from 10 river locations; water samples from all 10 and biofilm samples from two sites per grouping (LP: Less polluted; HP: Highly polluted).

samples within the LP group (Sites 6 and 7) were closely grouped; however, this is in contrast to the distance between the two matrices at Site 5 ("5" and "5B", Figure 5), with the biofilms at the HP sites visually much more abundant with increased density. This could indicate the establishment of biofilm communities that diverged from their planktonic counterparts. Nutrient composition has an influence on microbial community composition; thus, the abundant and dense biofilm establishment at Site 5 may emphasise nutrient differences between the water and biofilm matrices causing further community differences (Qu et al. 2017; Nawaz et al. 2018). Besemer (2015) also noted a lack of similarity between planktonic and biofilm communities from the same stream locations. The results from this study suggest that pollution may have a stronger impact on community composition than the planktonic versus sessile lifestyle. This could be explained by the characteristics of the extracellular polymeric substances (EPS) in the biofilm matrix; specifically, the entrapment and retention of pollutants (Mangwani et al. 2016; Mishra et al. 2022). This would be expected to exert a similar evolutionary pressure on the consistently polluted surrounding water columns. The potential for river biofilms to produce communities tolerant of constituents to which they are continuously exposed, together with the contrasting pathogenic profile observed between the biofilm and water samples of these two polluted sites, indicates the value of investigating both river matrices (Zegzouti et al. 2020; Tamminen et al. 2022).

3.3.2. Community acclimatisation

With respect to community differences before and after the acclimatisation experiment, and between media, there were clear shifts within all bioreactor groupings (Figure 6). Furthermore, community profiles differ between triplicate reactors, indicating the extent to which microbial communities can fluctuate within a limited period under selective pressure, despite similar composition and identical maintenance parameters. However, in support of the hypothesis proposing prior acclimatisation of the HP group, particularly in relation to the maintenance of filtered river water, these particular communities remained the most similar to their original composition. Moreover, the LP river water reactors showed the strongest grouping of triplicates and

also shifted less than the minimal medium reactors, indicating the feasibility and benefits of testing realistic bioreactor setups when investigating the use of environmental communities autochthonous to similar surroundings.

The variation in C:N ratios (data not shown) may have played a role in the observed community shifts, as seen in other studies (Huhe et al. 2017; Ni et al. 2018). The major shifts that occurred in the minimal medium reactors may also have been influenced by elevated N levels, added to the medium as ammonium sulphate ($[NH_4]_2SO_4$), which has been observed to have a significant effect on fungal community composition (Nawaz et al. 2018; Zhang et al. 2019; Ma et al. 2020; Yan et al. 2022).

3.3.3. Physico-chemical factors

Significant correlations between fungal community composition and physico-chemical parameters were observed (Figure 7). The strongest association was with TOC ($P = 0.001$), followed by N and P levels ($P < 0.01$), and COD and pH ($P < 0.02$). Fungi, as opposed to bacteria, demonstrate superior adaptation abilities when subject to stressors from environmental flux including extreme pH, inhibitors, low nutrients or the presence of toxic chemicals (Zegzouti et al. 2020). Therefore, parameters such as limited N or elevated COD levels are likely to drive shifts in fungal communities towards species with advantageous nutrient assimilation strategies. Qu et al. (2017) detected changes in stream water and biofilm communities closely correlated to nutrient levels. Chen et al. (2018) similarly detected significant impacts on the distribution of planktonic fungal profiles in response to COD and N fluctuations.

The standard physico-chemical parameters (phosphate, COD, nitrogen, TOC and pH) accounted for a large percentage of community variation (Figure 7); however, another driver of community shifts could be the MP content. This may be of particular note for the filtered river water reactors, considering that this medium is intended to reflect the environment from which they originate; moreover, the minimal medium reactors displayed much stronger correlations with the physico-chemical parameters discussed above. Tamminen et al. (2022) investigated community responses to wastewater exposure in stream biofilms, observing changes that were significantly associated with MPs, for both bacteria and eukaryotes. Determinants in diversity and community
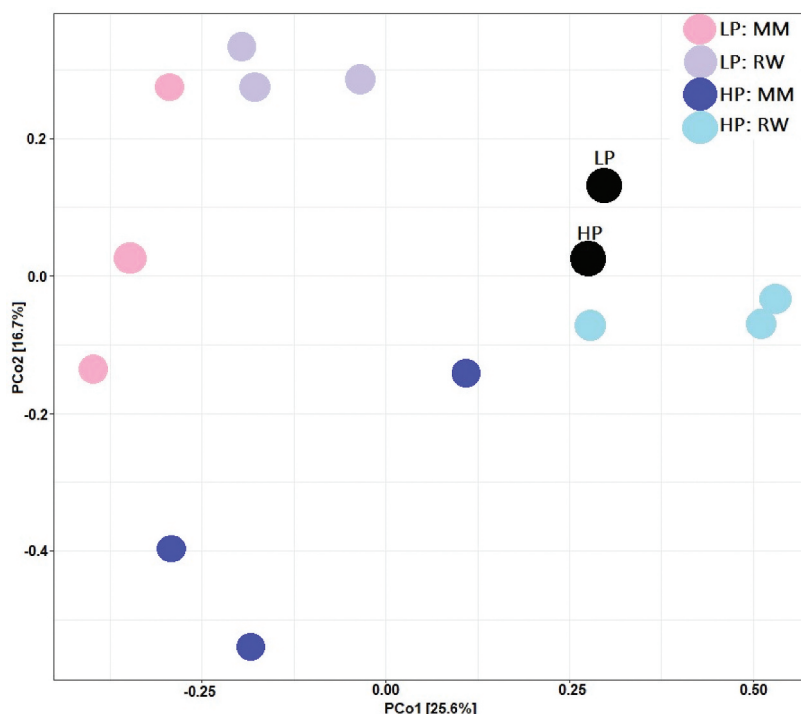
Figure 6. Principal coordinate analysis plots (PCoA) of fungal communities from environmental samples grouped into two initial communities (LP: Less polluted; HP: Highly polluted) and the (triplicate) results after a 7-week acclimatisation receiving either filtered river water (RW) or minimal medium (MM).
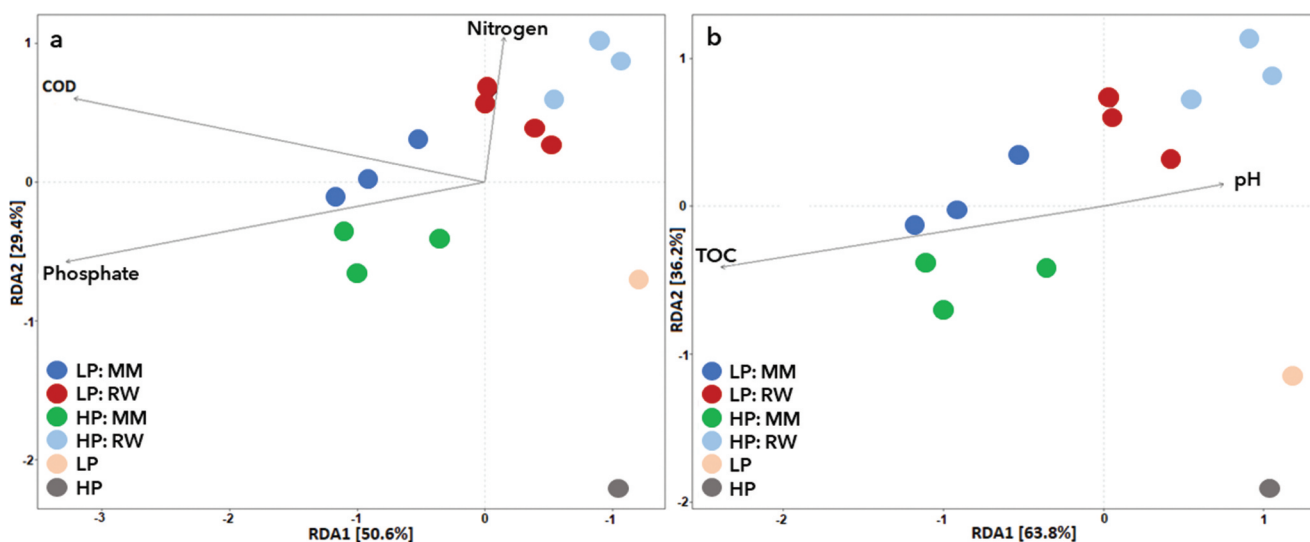
Figure 7. Redundancy analysis (RDA) of parameters significantly correlated to community composition from 12 bioreactors. LP: Less polluted; HP: Highly polluted; MM: Minimal medium; RW: River water medium. (a): Phosphate, COD and nitrogen. (b): TOC and pH.

composition remain challenging to establish, yet other studies have also detected the unique influence of MPs on microbial community structures (Harb et al. 2016; Phan et al. 2016). Moreover, a study by Tamminen et al. (2022) noticed an elevated response of eukaryotes, in particular, to wastewater influences. The river water was collected weekly to reflect

environmental variation; however, the processing may have caused natural degradation of the MPs present, thus contrasting with a river niche subject to the constant replenishment of MPs.

Although the bioreactor communities displayed clear shifts from their original compositions, Figure S4 illustrates the formation of a core community

that constituted 53.3% of all four reactor communities. Tamminen et al. (2022) observed stream communities to vary strongly locally, yet this was only apparent at species level and broader phylogenetic-level distribution agreed with Besemer (2015) and Saunders et al. (2016), both studies noting the establishment of abundant genera that commonly occur in WWTPs and stream biofilm communities, respectively.

Figure 8 and the diversity indices (Table 2) highlight the community shifts and overall reduction in diversity from the river to the laboratory in the absence of constant microbial re-supplementation from the water column, as observed by Shah et al. (2021). Although there is generally metabolic redundancy in microbial communities, the species dynamics and interdependent interactions of microbial communities are sensitive to abiotic change; thus, fluctuations in nutrient levels (such as N, P or oxygen)

and the adjustment to long media retention times in bioreactors can also induce major shifts in community structures (Abreu et al. 2019; Ruprecht et al. 2021; Shah et al. 2021).

Most of the minimal medium reactors resulted in a prevalence of the ascomycetous family *Dipodascaceae* (Figure 8). Two other dominant taxa after 7 weeks were the basidiomycetous *Apiotrichum* and *Cutaneotrichosporon* from the family *Trichosporonaceae*, reported to include physiologically diverse yeasts that assimilate aromatic compounds (Aliyu et al. 2020). The former genus is typically found environmentally, in water, rotten wood and food sources, whereas the latter is more commonly associated with humans (James et al. 2016; Aliyu et al. 2020). Proteome analyses of 33 members from the *Trichosporonaceae* family suggest functional diversification that correlated with isolation niches
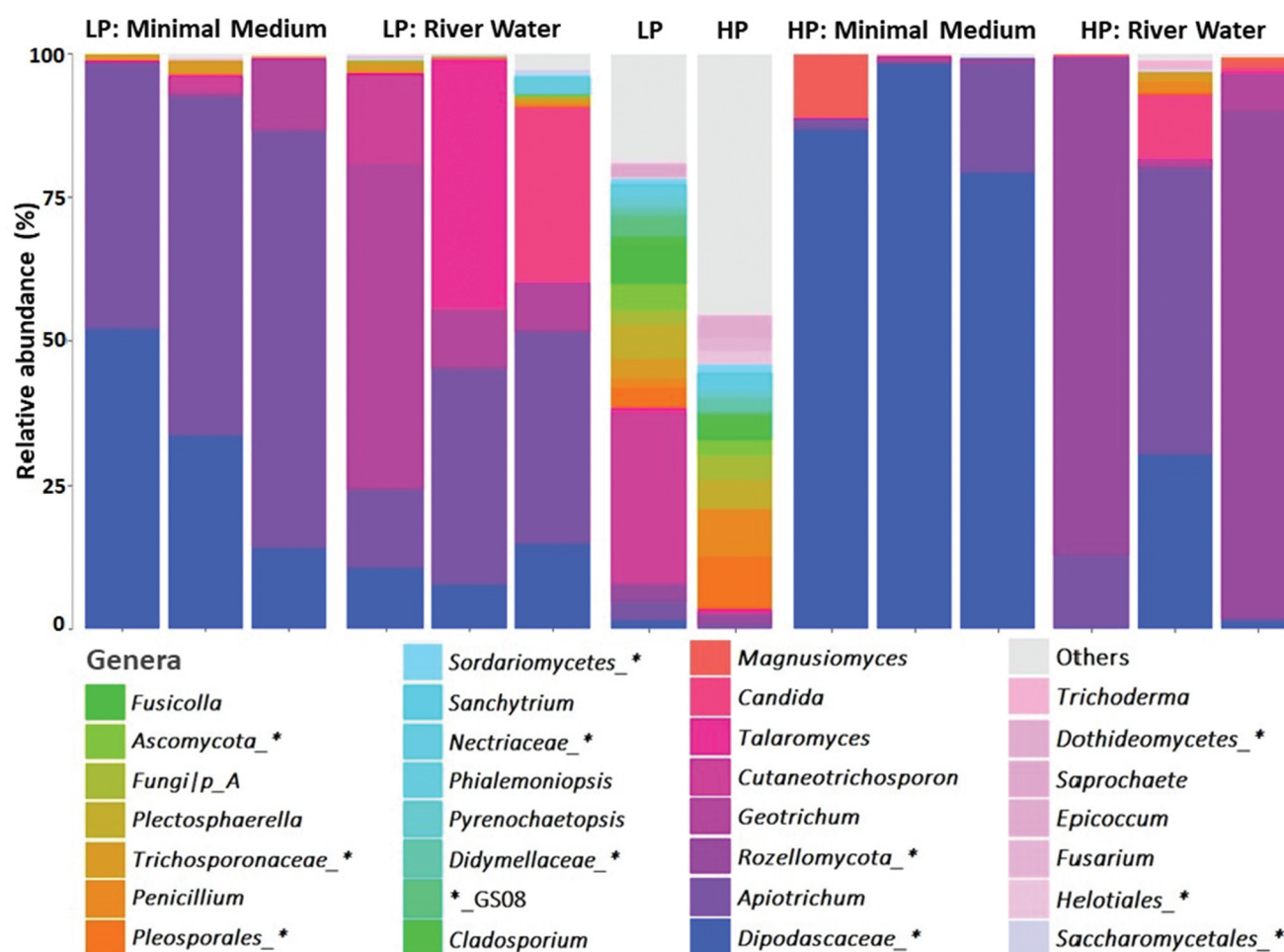
Figure 8. Relative abundance of most dominant genera in the initial combined inocula (LP/HP) and the final communities after maintenance for 7 weeks on filtered river water or minimal medium (each bioreactor type in triplicate). _*: Unclassified genus (preceded by highest taxonomic identification).

Table 2. Alpha diversity indices (means) from 10 river sites before and after 7 weeks in bioreactors.

Site	Coverage (%)	Shannon	Simpson	InvSimpson	Chao1	ACE	Fisher
1–5 (HP)	82 ± 0.0	5.0 ± 0.8	1.0 ± 0.1	57.5 ± 32.1	826 ± 140	937 ± 160	238 ± 77
6–10 (LP)	79 ± 0.1	5.3 ± 0.9	1.0 ± 0.1	96.3 ± 52.9	949 ± 280	1063 ± 321	325 ± 122
HP inoc.	65	5.8	1.0	118.9	1314	1503	451
HP[1]	97 ± 0.0	1.5 ± 1.2	0.5 ± 0.3	3.5 ± 3.4	89 ± 39	113 ± 75	15 ± 12
HP[2]	99 ± 0.0	1.0 ± 0.7	0.4 ± 0.3	2.6 ± 1.1	45 ± 21	105 ± 41	8 ± 5
LP inoc.	81	4.3	0.9	16.5	703	842	158
LP[1]	95 ± 2	1.7 ± 0.3	0.6 ± 0.1	2.7 ± 0.6	185 ± 63	232 ± 90	21 ± 8
LP[2]	96 ± 2	2.0 ± 0.4	0.7 ± 0.1	4 ± 1.3	171 ± 61	199 ± 78	19 ± 10

[1]Reactors maintained with minimal medium (highlighted). [2]Reactors maintained with filtered river water. LP: Less polluted; HP: Highly polluted; InvSimpson: Inverse Simpson; ACE: Abundance-based coverage estimator.

and respective substrate differences; thus, biotechnological application has been proposed for oleaginous species from both the aforementioned genera (Aliyu et al. 2020).

Two further dominant genera were *Geotrichum* and an unclassified member of *Rozellomycota* (*Cryptomycota*), a clade exhibiting high phylogenetic diversity in aquatic and terrestrial habitats together with an obligate pathogenic dependency on various eukaryotes such as amoebae or algae (Tedersoo et al. 2017). The abundance of *Geotrichum* and *Trichoderma* increased over the acclimatisation period, particularly in the river water reactors, several species of which have been recognised for their potential in the transformation of xenobiotic compounds (Aranda 2016; Vaksmaa et al. 2023). Select species of *Trichoderma* and *Geotrichum* have been utilised for a variety of remedial applications, including but not limited to the degradation of veterinary and human pharmaceuticals, heavy metals and plastics (Parshikov et al. 2002; Asses et al. 2009; Dragičević et al. 2010; Bouchiat et al. 2016; Vaksmaa et al. 2023). The survival and even persistence of the aforementioned genera during the experimental period, despite a lack of optimal fungal conditions, indicates the value of screening environmental communities using realistic and autochthonous parameters, and the potential feasibility of riverine taxa for xenobiotic degradation if evolutionary pressures in the bioreactors select for capable fungi.

3.4. Diversity

Select α-diversity indices are summarised in Table 2, including the means of (1) the communities sampled from 10 river sites individually, (2) two combined inocula after grouping Sites 1–5 (HP) and Sites 6–10 (LP), and (3) communities isolated from the four bioreactor groupings, post-acclimatisation.

The diversity indices of the HP and LP groups (five sites/seven samples each) were not significantly different from each other (Kruskal-Wallis; *P* values 0.06 to 0.14), likely due to high variance between sites within the two groups (Table 2, standard deviation). However, the combined HP inoculum was significantly more diverse than LP for all indices except Shannon and Simpson, which were still higher in the HP group, despite being not significant. This contrasts with the indices of the aforementioned individual sites that were all higher in the LP and not the HP group (except coverage), despite the combined inocula being weighted towards the biofilm samples that both showed higher diversity in the LP group too. These results from the individual samples (particularly biofilm), although not significant, correlate with observations of reduced benthic biofilm diversity in a nutrient-rich urban stream (assumed to contain more pollution) compared to more pristine ones (Besemer 2015). However, this also indicates how sample processing can affect the results of community analyses extensively, although not predictably. It is expected that low concentrations of DNA, likely representing less abundant species, may be masked by the DNA of more abundant species, thus decreasing diversity results. However, while the LP group's diversity decreased when analysed as a combined inoculum, the HP indices all increased substantially, indicating the importance of analyses at each experimental step to avoid potentially inaccurate assumptions.

Each of the four bioreactor groupings displayed significantly lower community diversity than their original inoculums across all indices except

Simpson, which also decreased although not significantly. Interestingly, there were no significant differences between the final communities of the four reactor groupings, i.e. all communities converged towards similarly low values (in comparison to their original diversity) for all indices. Artigas et al. (2012) studied biofilm colonisation in two contrasting rivers, with differing nutrient levels (NH_4^+, NO_3^- and P) and only one that was subject to flooding events. Although colonisation patterns (i.e. increases in species richness) varied between the rivers in the first few weeks, by the 44th day they were almost identical and after 60 days the community composition remained very similar in terms of biofilm structure and metabolic complexity. In this study, the minimal medium contained more C, N (in the form of NH_4^+ and amino acids) and P, yet the diversity of these communities decreased similarly to the river water reactors over the 49 days. The river niches that harbour the sampled communities are subject to a continuous flux of both nutrients and, possibly more notably, microorganisms. Flux, or instability, has been shown to increase diversity, also observed by Artigas et al. (2012) whereby flooding events caused an increase in diversity due to the disruption of succession followed by re-colonisation, which may favour the re-appearance of early colonisers. However, the river water used as a medium was changed only weekly and filtered to prevent continuous re-inoculation. Therefore, it is possible that microbial communities respond to environmental shifts by rapid community transitions in natural open systems by maintaining (or increasing) diversity, whereas a controlled environment (such as the reactors) may result in overall diversity reduction once initial community shifts have occurred. This could also contribute to the overall lower diversity of the biofilm matrix, due to attachment and increased residence time, in comparison to its planktonic counterparts.

When considering the decline in diversity over the 7 weeks, comparing both LP and HP inocula to the two bioreactor types below each, supports the assumption that acclimatisation and adaptation to surrounding nutrients may drive diversity shifts. The HP communities, accustomed to high organic loads including carbon, experienced more diversity decreases in the river water reactors that contained

fourfold lower carbon than the minimal medium. For the LP communities, a larger reduction in diversity was observed for the minimal medium reactors, again the medium presumed to be most contrasting with their typical niche of cleaner river water containing low levels of nutrients. Furthermore, these results support an alternate hypothesis; namely, that limited nutrients drive the dominance of a limited number of capable species, thus decreasing diversity indices. However, the term nutrients is broad and refers to a multitude of factors that may alter community composition and diversity, the mechanisms of which remain unclear (Besemer 2015). Johnson et al. (2015) and Wolff et al. (2018) noted an inverse correlation between taxonomic richness, and C and N availability, although only bacteria (of mixed communities) were sequenced. Ni et al. (2018) and Di Lonardo et al. (2020) observed increased fungal richness and growth efficiency, respectively, correlated to lower C:N ratios in soil, i.e. higher N availability. A review by Drost et al. (2021) also supported the hypothesis that fungal diversity decreases when substrates have higher C:N ratios. Such a food source is more difficult to degrade, requiring specialists that invest high energy to utilise recalcitrant compounds, thus concurrently preventing competition from other microbes by creating an unfavourable environment (i.e. antagonism via secondary metabolites), consequently reducing diversity. Overall, the C:N ratios for the two media in this experiment, using only TOC and TN results, were relatively high (>10), a possible explanation for the overall decrease in diversity according to the above studies.

Although biodiversity may be essential for facilitating ecosystem functions, diversity does not necessarily correlate with desirable characteristics for biological treatment; in fact, the results from this study may prove promising for future MP experiments. Liang et al. (2021) noted a lack of correlation between (bacterial community) diversity and biotransformation of MPs, and Kruglova et al. (2017) observed that increased (bacterial) diversity caused reduced MP removal potential of the microbial community. In contrast, Johnson et al. (2015) hypothesised that higher biodiversity may be required to maximise collective rates of multiple MP biotransformations, although on an individual MP level, this was

varied, and positive associations were stronger for rare MPs. Falås et al. (2018) demonstrated that MP uptake was performed by less than 0.1% of the microbial community and that these agents differed between compounds; moreover, the results pointed to a high level of microbial substrate specialisation that would cause differences in transformation characteristics between MPs and microbial communities. It is assumed that diversity is important for degradation, although this is debated (Drost et al. 2021). More specifically, functional diversity is being increasingly recognised as a more insightful indication of ecosystem processes (Besemer 2015; Tamminen et al. 2022). Thus, further research on the correlations between community structure and metabolic functioning would be beneficial for selecting potential bioremediation candidates.

4. Conclusions

There is concern that fungi pose an increasing human health risk due to rising environmental temperatures that favour opportunistic genera, persistent medical and agricultural antifungal use, and human populations containing high-immunocompromised numbers susceptible to opportunistic fungal agents. Routes of infection, aside from nosocomial, include direct contact with contaminated environmental waters, and exposure to inefficiently treated potable water.

Opportunistic fungal genera and pathogenic species were detected at several public river sites in the study area. Together with the correlation and constant exposure of these potentially infectious agents to high levels of pollution that could lead to increased pathogenicity or the production of toxic secondary metabolites, this microbial facet of water quality monitoring deserves further attention in countries with high urbanisation rates that lead to insufficient treatment capacity. This study also demonstrated the rationale for the incorporation of fungal species into water quality monitoring due to their correlations with faecal indicator organisms, a detectable yet transient presence in environmental water, and links to the human gut. Moreover, fungi are more resilient towards standard disinfection practices; thus, an absence of standard water quality indicator organisms could be an overestimation of water safety due to the current exclusion of fungal representatives. Further research into species from the genera detected in this study that possess promising characteristics towards use as indicator organisms is warranted.

While fungi potentially pose a serious human health threat in the context of climate change, they also possess promising capabilities towards biodegradation of xenobiotic compounds. Pharmaceuticals, personal care products and other organic chemicals, collectively termed MPs due to their persistent yet typically low-level presence in environmental waters, are a constant burden on water quality in terms of human and environmental health as a result of cumulative or mixture toxicity. Environmentally- and cost- friendly methods for the biodegradation of MPs as a critical step in water treatment are continuously being developed, with promising results obtained using fungi and their superior enzymes. The intrinsic ability to acclimatise observed in this study demonstrates the survivability of environmental fungi. Together with the observed decrease in almost all detected fungal pathogens over the acclimatisation period, such results support the feasibility of environmental communities containing fungi for use in further research towards improved environmental stewardship.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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