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Environmental significance statement

Fungi are being increasingly acknowledged as emerging microbial contaminants that are present in several environmental compartments. Pathogenic fungi, in particular, are of growing concern, partly due to the disproportionate risk that they pose to immunocompromised individuals. A parallel reason for this increased concern is the effect that climate change is having on the geospatial distribution of fungal pathogens. This study characterizes fungal pathogens in irrigation canal waters impacted by wastewater discharge and/or aerosol emissions from aeration basins and other potential anthropogenic sources. The importance of this work lays in evaluating the possible impact of different environmental factors (wind, temperature, humidity, stream flow, etc.) on dispersal and transport of fungal populations and their associated pathogens from these anthropogenic sources.

Fungi as an emerging waterborne health concern: Impact of treated wastewater discharge *versus* aerosolization

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Abstract

The discharge of treated wastewater effluents into river-fed irrigation canals results in a de facto form of water reuse. Waterborne fungal populations in such environments pose a unique human health concern given that opportunistic fungal pathogens can be proliferated during spray irrigation of crops. In the present study, we consider two different routes (effluent discharge *versus* bioaerosols) through which wastewater treatment plants (WWTPs) can impact the presence and abundance of fungal communities in irrigation canals of the Rio Grande river basin in New Mexico. Site A was selected to investigate the influence of effluent discharge from a WWTP on waterborne fungal communities in a receiving irrigation canal. Site B represented an irrigation canal that was directly adjacent to a WWTP but that receives no effluent discharge (to exemplify bioaerosolization exclusively). Sampling dates were chosen to capture variations in weather and stream flow conditions at each of the two sites. Results indicated that treated wastewater discharged into the canal had a distinct impact on fungal community composition, especially under low wind and flow conditions. When stream flow was highest, variations along the canal at Site A were minimal. The highest occurrence of pathogen-associated genera was observed at Site B under high wind conditions with an average relative abundance of 20.9 ± 13.1% (peak of 39.3%) and was attributable to bioaerosol emissions from the WWTP and a nearby livestock facility. Such genera included *Alternaria*, *Cladosporium,* and

Cryptococcus. These findings suggest that although treated effluent discharge can directly impact irrigation canal fungal community composition, bioaerosols likely have a larger overall effect on the spread of potential fungal pathogens.

Keywords: fungal, pathogen, public health, effluent, bioaerosols, water reuse

1. Introduction

Increasing droughts and water scarcity have intensified the reliance on surface waters that are heavily impacted by wastewater discharges. This results in various de facto forms of indirect water reuse.[1] The Rio Grande, one of the principal rivers in the Southwest region, is a main water resource for agricultural activities that include spray irrigation of crops. Wastewater treatment plants (WWTPs) and other facilities that surround Rio Grande-associated irrigation canals produce discharges and emissions that can introduce a range of potentially harmful microbes to their waters. One of the more scarcely studied groups among such microbes are waterborne fungi.

Fungi are ubiquitous components of all ecosystems, including aquatic environments.[2] With surface waters (e.g. rivers, lakes) serving as conventional water resources, their evaluation as fungal habitats is of broad interest. Anthropogenic releases associated with wastewater treatment (among other industrial activities) can serve as point sources of waterborne fungi, some of which are known to be harmful (i.e., pathogens).[3-7] The presence of these fungi can increase human health risk, especially when irrigation is a primary use of affected waterways. Fungal pathogens are acknowledged as an emerging human health concern, yet they remain largely overlooked in water environments. Waterborne fungi that were previously considered non-pathogenic are increasingly being associated with human disease.[8] Recently (and for the first time), the World Health Organization (WHO) has released a list of fungi as priority pathogens that includes species of *Candida*, *Cryptococcus*, and *Aspergillus* owing to recent outbreaks and acute risk to immunocompromised individuals.[9] Aside from their opportunistic nature, public health concerns towards fungal pathogenicity have evolved due to increases in resistance to certain antifungal classes over time. For example, *Aspergillus fumigatus* has recently seen a spike in rates of azole resistance, while many *Candida*

auris strains are showing resistance to all three major classes of antifungal drugs (azoles, echinocandins, and polyenes).[10, 11] This reality will be further exacerbated in coming decades by the increased use of antifungals in both agricultural and clinical settings.[12, 13]

Overall, water environments remain vastly understudied in their role as fungal habitats. Water environments such as supply reservoirs, hospital hot water systems, swimming pools, marine sediments, and wastewaters can act as pools for the growth and spread of fungi.[14-17] Among those, wastewater has been shown to harbor specific fungal groups identified as opportunistic pathogens.[3,5,17,18] *Aspergillus*, *Penicillium*, and *Cladosporium* are genera of particular interest given their high resistance to disinfection in wastewater treatment systems.[19,20] The discharge of treated effluents into open water bodies such as rivers and irrigation canals increases the potential presence of fungal pathogens in streams. Species of *Penicillium*, *Candida*, and *Geotrichum*, identified as human pathogens,[18,21-23] have been frequently found in treated wastewaters.[23,24] Based on this, the persistence of waterborne fungi that have health implications requires further investigation to help predict downstream contamination and assess potential effects of human exposure.

In addition to contamination through treated effluent discharges, fungi and other microorganisms can be released into the atmosphere via aerosolization from different stages of wastewater treatment[25-28] as well as from other relevant facilities (e.g., dairy farms and other animal operations).[6,7] Inhalation is the most common route of human exposure to fungal pathogens. For example, infections can occur from waterborne fungi by inhalation of water droplets originating from contaminated water sources during spray irrigation of crops.[29] Characteristics of fungi often accentuate their capacity to aerosolize. For example, the formation of fungal spores increases transfer rates into the atmosphere and, ultimately, the chances of human infection. These spores also have the capacity to act as reservoirs of mycotoxins, causing additional health hazards associated with the inhalation of bioaerosols.[29-31] Environmental factors such as temperature, relative humidity, wind speed, and rainfall events have all been found to contribute to the airborne viability and concentration of fungi.[32, 33] Therefore, the use of potentially contaminated surface waters for irrigation raises

concerns of human exposure to airborne fungi.[34] Frequently occurring aerosolized fungi from WWTPs include species of *Cladosporium*, *Candida, Mycelia*, *Penicillium*, and *Rhodotorula*.[35] Airborne presence of such groups is typically concentrated near the aeration basin, with some potentially being carried downwind with sufficient wind speed and dispersion.[25,27,28] Fungal spores of *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, and *Rhizopus* can be easily carried with winds and have been observed at high abundances nearly 200 m downwind of a dairy farm lot.[33]

Traditionally, treated wastewater effluent safety has been evaluated through quantitative assessment of indicator microorganisms (i.e., *Escherichia coli*, enterococci, fecal coliforms, etc.). However, these methods tend to be biased and underestimate pathogenic risks as they do not specifically target any form of fungal or emerging pathogen.[36, 37] Alternative methods ought to be developed and integrated to account for the presence of opportunistic fungal groups in aquatic environments. Quantitative assessments are therefore needed in order to estimate the plausible proliferation of fungal pathogens in wastewaters and surface waters alike.[16] The quantification of fungal pathogens in such settings will improve the surveillance of emerging pathogens in wastewaters and, ultimately, the prevention of outbreaks. This is an essential step towards meeting public health protection goals that are in line with global initiatives such as GLOWACON.[38]

To identify sources potentially contributing to fungal contamination of surface water resources along the Rio Grande, the sampling campaign for this project encompassed two distinct areas along the irrigation canals of the Rio Grande river basin in central New Mexico: Site A, where a canal is directly receiving the effluent discharge of a WWTP, and Site B, consisting of a canal affected by aerosols (but not discharge) of a WWTP and a livestock facility. The main objective was to evaluate the potential effects of (1) treated wastewater discharges and (2) aerosolization of fungi from WWTPs and other facilities on the presence and persistence of fungal communities (and their associated potential pathogens) in irrigation canals. Such an assessment of fungal populations in water bodies affected by anthropogenic sources is a step towards improving understanding the emerging risks of fungal pathogens.

2. Materials and methods

2.1. Sampling procedure

The sampling campaign focused on irrigation canals along the Rio Grande river basin in central New Mexico. Sampling locations were chosen to reflect the impact of a treated wastewater discharge (Site A) and aerosol discharge from a WWTP and other nearby facilities (Site B) on the fungal community composition in the irrigation canals. The WWTP discharging at Site A treats an average daily influent flow of 0.8 million gallons per day (MGD) with an activated sludge-based sequencing batch reactor (SBR). For Site A, samples were collected from the canal 200 m upstream of the WWTP outfall (A1), at the discharge point of the outfall (A2), and 500 m downstream of the outfall (A3). The WWTP at Site B treats approximately 0.9 MGD, also using activated sludge in the form of a membrane bioreactor (MBR). The WWTP of Site B does not discharge its effluent into the adjacent canal, but to the Rio Grande directly. For Site B, samples were collected from the canal 2500 m upstream (B1), 350 m downstream (B2), and 1000 m downstream of the WWTP (B3). A livestock facility is also located near Site B approximately 1100 m downstream of the WWTP. Sampling locations for both sites are shown in Figure 1.

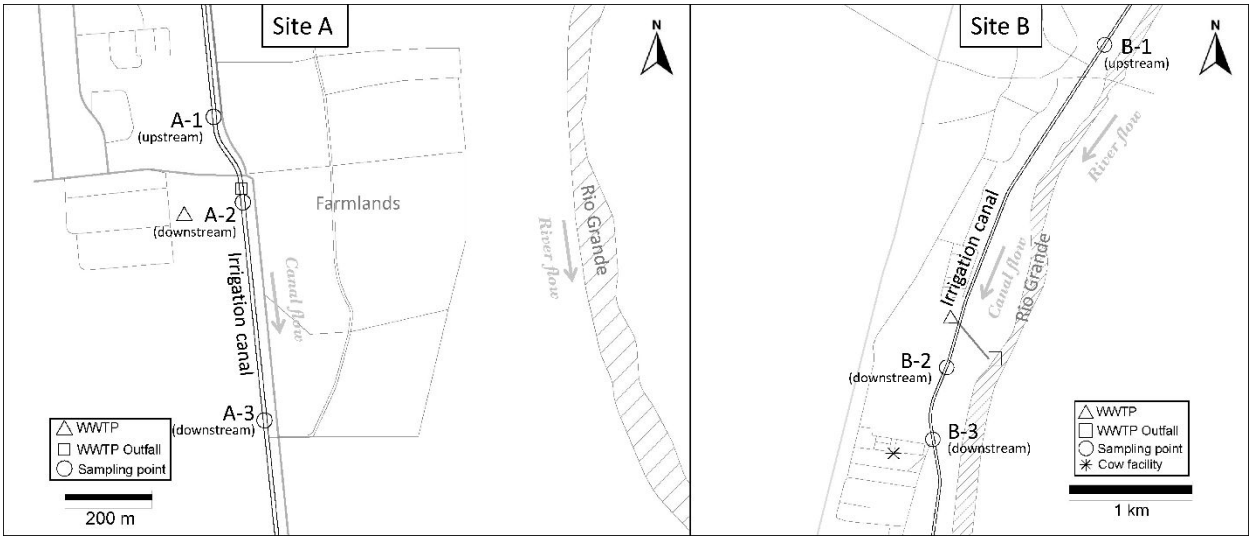

Figure 1 Sampling points along irrigation canals at Site A (A1 through A3) and Site B (B1 through B3). Sampling points A1, A2, and A3 correspond to samples taken 200 m upstream, at the outfall point, and approximately 500 m downstream of the outfall, respectively. Sampling point B1 is located more than 2500 m upstream of the WWTP, B2 350 m downstream, and B3 at the edge of a livestock facility that is 1000 m downstream of the WWTP.

Sampling took place on three separate dates while ensuring the absence of rainfall events at least 5 days prior to sampling. Water sampling dates were set with 2-month temporal separation. Samples were collected in 10-20 L polypropylene carboys, transported to the laboratory within 1.5 h of collection, and stored at 4 °C until further processing and water quality analysis. Wind, temperature, humidity, and stream flow data were retrieved from the National Weather Service (NWS) Western Region Headquarters and the National Water Information System (NWIS) of the USGS and used for evaluation of results (Table 1). As exact stream flow rates were not known for the irrigation canals themselves, stream flow data for the Rio Grande was used as a proxy. A matrix was set to help evaluate the effect of surrounding facilities on the irrigation canal waters assuming that stream flow, wind speed, and wind direction were primary influencing factors (Figure 2). In general, it was anticipated that high stream flow would increase mixing and transport (i.e., dilute effects of treated wastewater discharge) while high winds and/or wind in the direction of the canal could increase aerosol impacts.

Table 1 Weather conditions and stream flow values near each of the sampling Sites A and B. T refers to air temperature in °C, RH refers to relative humidity in %, w.s. refers to wind speed in kilometers per hour (kph), w.d. refers to wind direction, and Q refers to stream flow rate in cubic meters per second (m^3/s).

	August					October					December				
	T	RH	w.s.	w.d.	Q	T	RH	w.s.	w.d.	Q	T	RH	w.s.	w.d.	Q
Site A	29	31	10	ENE	2.8	21	28	19	SSE	2.8	7	65	5	N	58.1
Site B	31	26	5	SSW	2.3	25	18	26	SSW	2.8	10	40	5	SE	53.8

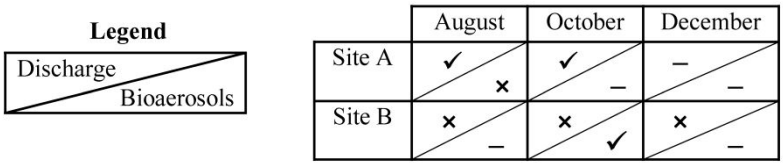

Figure 2 Matrix showing predicted influencing factors on fungal microbial composition at each of the sites investigated. The top left of each cell shows anticipated impact of effluent discharge while the bottom right shows anticipated impact of bioaerosols. × indicates no predicted impact, – indicates possible/limited impact, and ✓ indicates likely impact.

2.2. Water quality testing

Water quality parameters were measured in duplicate for all samples taken. Samples were stored at 4 °C until measurements were made. pH, turbidity, and conductivity were measured using a pH meter,

turbidimeter, and conductivity meter, respectively. Total suspended solids (TSS) was tested following APHA Standard Method 2540.[39] Total dissolved solids (TDS) was calculated based on TSS and total solids (TS) concentrations. TS was determined by evaporating an 80 mL water volume in a 105 °C oven over 8 hours. Chemical oxygen demand (COD) was tested according to the USEPA Reactor Digestion Method. Ammonia, nitrate, nitrite, and phosphorus were tested using TNTPlus vial kits (Hach, USA). Absorbance values used to determine chemical water quality parameters were measured using a Spectronic Genesys 5 Spectrophotometer at the corresponding wavelengths (Milton Roy, USA). Concentrations were subsequently determined using generated standard curves. All tests were performed within one week of the sample collection date.

2.3. Fungal community characterization

Between 0.8 and 3.0 L of each collected sample was filtered through a 0.22 μm mixed cellulose ester (MCE) membrane filter (in triplicate) and stored at -20 °C prior to processing. Filtration was completed within three days of sample collection. Total DNA was extracted from the filters using the DNeasy PowerSoil Pro Kit (Qiagen, USA). DNA concentration and quality were measured on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples were sequenced at an external laboratory (MR DNA, Shallowater, TX, USA) after amplification of the internal transcribed spacer (ITS) region of the genome. The primer set ITS1F/ITS2 (forward and reverse) targeting the ITS1 sub-region was used to characterize fungal communities.[40-42] Amplicons were multiplexed and sequenced on the Illumina MiSeq platform following manufacturer's guidelines using 300 bp paired-end reads. Other details on sequencing procedures and primers used are provided in the supplementary information (SI).

Sequences were processed and classified into operational taxonomic units (OTUs) using MR DNA's internal analysis pipeline. Unique sequences were obtained by eliminating those with ambiguous calls or those less than 150 bp. Sequence clustering was based on a 1.0 maximum expected error threshold and was followed by chimera removal. The resulting zOTUs (zero-radius OTUs) were taxonomically classified by blasting the sequences using BLASTn against a corresponding ITS database derived from the National

Center for Biotechnology Information (NCBI). Obtained classifications were used to generate relative abundance charts and also to identify potentially pathogenic fungal groups. Further data analysis was performed on the Mothur bioinformatics platform[43] to elucidate similarities/differences between samples utilizing alpha and beta diversity measurements based on the Schloss MiSeq SOP.[44]

2.4. 18S rRNA gene quantification

Extracted DNA samples were also used for absolute quantification of total fungal abundance using quantitative PCR (qPCR). The 18S rRNA gene was targeted by qPCR to determine total copy number per volume of sample. Primers were selected to minimize potential amplification of non-target sequences. qPCR standards were developed from a *Saccharomyces cerevisiae* culture (VWR, USA) and amplified using the primer set NS1/Fung.[14,45,46] qPCR was performed on a qTOWER^{3}G system (Analytik Jena, Germany) with sample plates prepared using Forget-Me-Not qPCR master mix (Biotium, USA). 20 µL reactions consisted of 10 µL master mix, 1 µL of each forward and reverse primers at 10 µM, 1 µL of template, and 7 µL of molecular grade water. Details on preparation and growth of *Saccharomyces cerevisiae* and generation of qPCR standard curves are provided in SI. Primer sequences and thermal cycling conditions are shown in Table S2.

2.5. Statistical analysis

Statistical differences between samples were evaluated using an unpaired two-tailed t-test, assuming unequal variance between sample sets. Correlations were established between measured water quality parameters and fungal groups using a calculated Pearson correlation coefficient (r) and multi-linear regression (MLR) analysis. The Pearson coefficient, r, correlating individual parameters and fungal populations was first determined in Microsoft Excel using the PEARSON function. Variables showing correlations of $r > |0.6|$ were used for direct analysis (Table 2). Weaker correlations ($|0.3| < r < |0.6|$) were taken as a reference for further evaluation using MLR. A single dependent variable was then selected and correlated with several parameters taken as independent variables. Regression analysis was performed using the regression data analysis tool in Microsoft Excel and subsequently considered for analysis for cases

yielding an overall confidence interval > 95% (i.e., $F < 0.05$). This was used to assess the combined effect of multiple parameters on a single variable with each parameter having an individual coefficient and significance of correlation with the dependent variable (Table 3).

3. Results and discussion

For the two sites under investigation, distinct aspects of environmental conditions are expected to influence fungal community variations along the canal. At Site A, treated wastewater discharge is the predominant contributor, with the effect being lower under high stream flow conditions. Also at Site A, wind is expected to be a secondary contributor under high wind conditions and/or when wind direction is towards the irrigation canal. At Site B, wastewater discharge has no impact (no outfall), and no changes are anticipated in non-microbial water quality parameters. Any anthropogenic impact on fungal communities at points B2 and B3 are expected to result from bioaerosol discharge and deposition from either the WWTP or livestock facility, with wind speed and direction playing a key role.

3.1. Changes in irrigation canal water quality

Variations in water quality were the first clear indicator of the impact of discharged effluents at Site A (locations A2 and A3). Nitrite and phosphorus concentrations peaked at 1.70 mg/L and 2.75 mg/L, respectively, at A2 in August while a spike in COD was seen in A3 (Figure 3). The COD spike appeared to be an anomaly and may have resulted from the proximity of A3 to secondary irrigation canal inflow (shown in Figure 1). Bioaerosol emissions from Site B had no observable impact on sampling points B2 and B3. Overall, ammonia (unpaired *t*-test, $P < 0.00001$) and nitrite (unpaired *t*-test, $P = 0.012$) concentrations were significantly higher in August compared to December, while nitrate was significantly higher in December than October (unpaired *t*-test, $P = 0.00019$).

Measured pH values were consistent across all samples, with an average of 8.0 ± 0.12. Turbidity dropped in sample A2 in August (shown in Figure S1), coinciding with the spikes in phosphorus and nitrite (Figure 3). Significantly higher turbidity was measured at Site B (73.7 ± 13.9 NTU) compared to Site A (unpaired *t*-test, $P = 0.04$). Conductivity was stable by location and consistently higher in Site A than Site

B samples with respective averages of 1003 ± 55 µS/cm and 505 ± 41 µS/cm. A similar trend was observed for solids concentrations (Figure 4), showing a positive correlation with conductivity (Table S3). Higher TSS concentrations at Site B in August resulted in significantly higher TSS/TDS ratios compared to Site A (unpaired *t*-test, $P = 0.002$), which in turn had a positive correlation with sample turbidity.

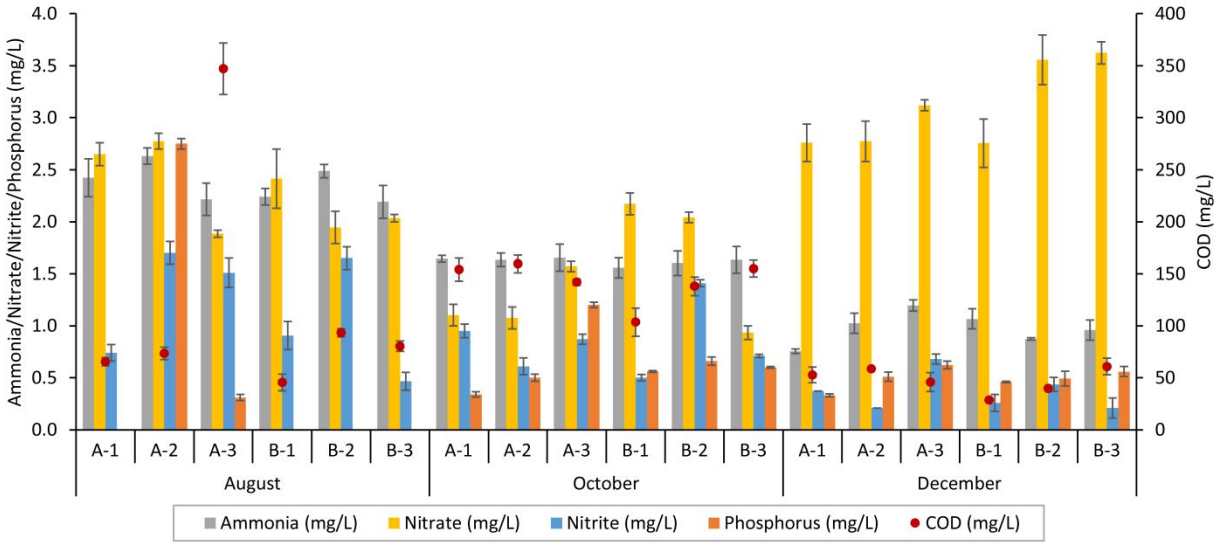

Figure 3 Chemical water quality parameters in August, October, and December for all sampling locations.

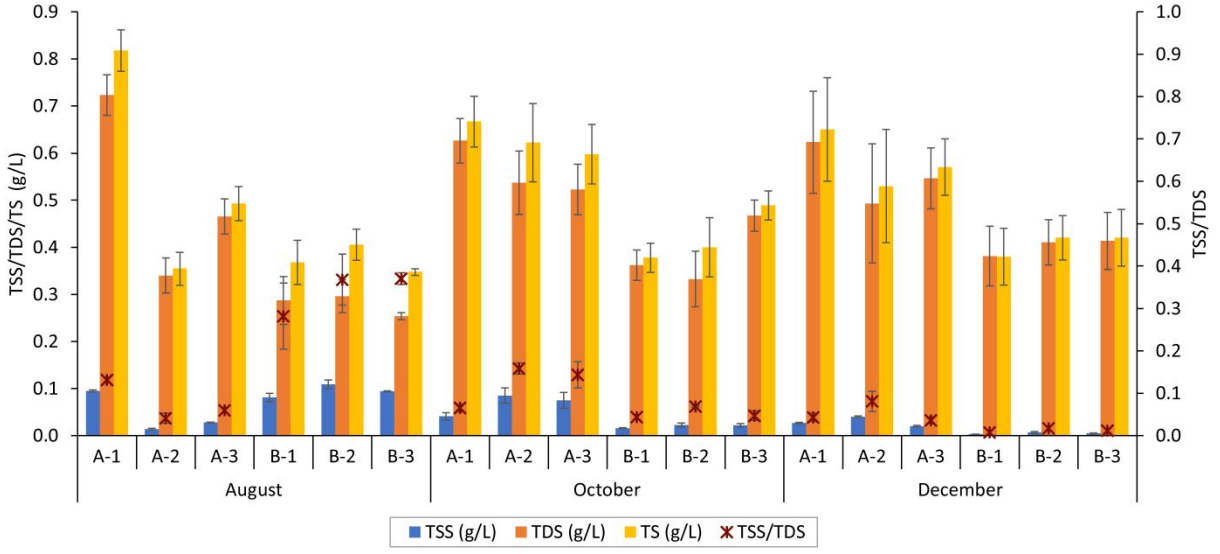

Figure 4 Concentrations of total solids (TS), total dissolved solids (TDS), total suspended solids (TSS), and TSS/TDS ratio for all water samples collected in August, October, and December.

3.2. Overall variations in fungal communities

Activated sludge-based WWTPs contain diverse fungal communities that vary by location and environmental conditions.[3,17] Previous research has shown that fungal compositions are typically prone to seasonal variations.[4,47,48] Bioaerosol emissions from WWTPs[27,28] and livestock operations[7,49] are also impacted by seasonally-imposed environmental factors such as temperature, relative humidity, and wind conditions. In the present study, such variations were reflected in the principal coordinate analysis (PCoA) (Figure 5). Clustering can be observed for points representing Site B for each sampling date, with notable shifts over the changes in season. This is attributable to both (1) fungal community affinities for specific temperature and relative humidity ranges and (2) differences in aerosol contributions to the background waterborne fungal community makeup.[34] Also notable was that Site A had a distinct profile at location A2 in August, with higher similarity between sampling points in October and December.

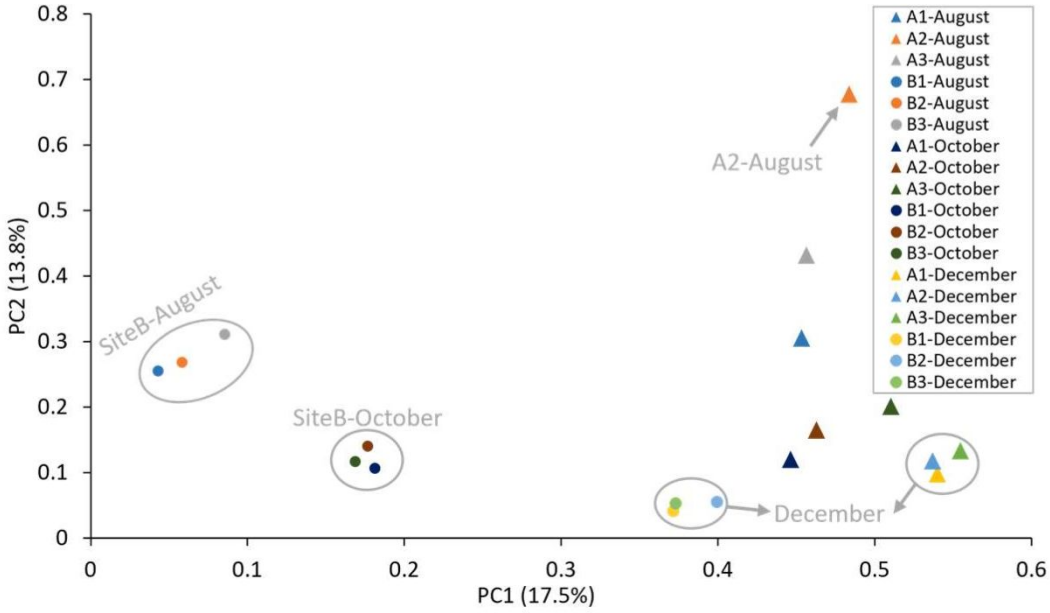

Figure 5 Principal coordinates analysis (PCoA) plot representing fungal community similarity for all water samples based on a Jclass similarity matrix using genus-level sequence clustering.

Relative abundance charts showed that the discharge from the WWTP at Site A had a major impact in August (also seen in the PCoA), having distinguished downstream (A2 and A3) dominant groups. *Sclerococcum* and *Pyrenochaetopsis* initially peaked near the discharge point (A2) at respective abundances

of 75.5% and 16.3% and were carried downstream to location A3. At the same time, Site B was dominated by unique fungal groups, including *Gaertneriomyces* and *Angulomyces*, undetected in Site A. Low wind and flow conditions may have contributed to the distinct impact of the WWTP outfall in August relative to the other two sampling dates. By comparison, water samples in October and December showed limited changes across sampling points at both Sites A and B (Figure 6).

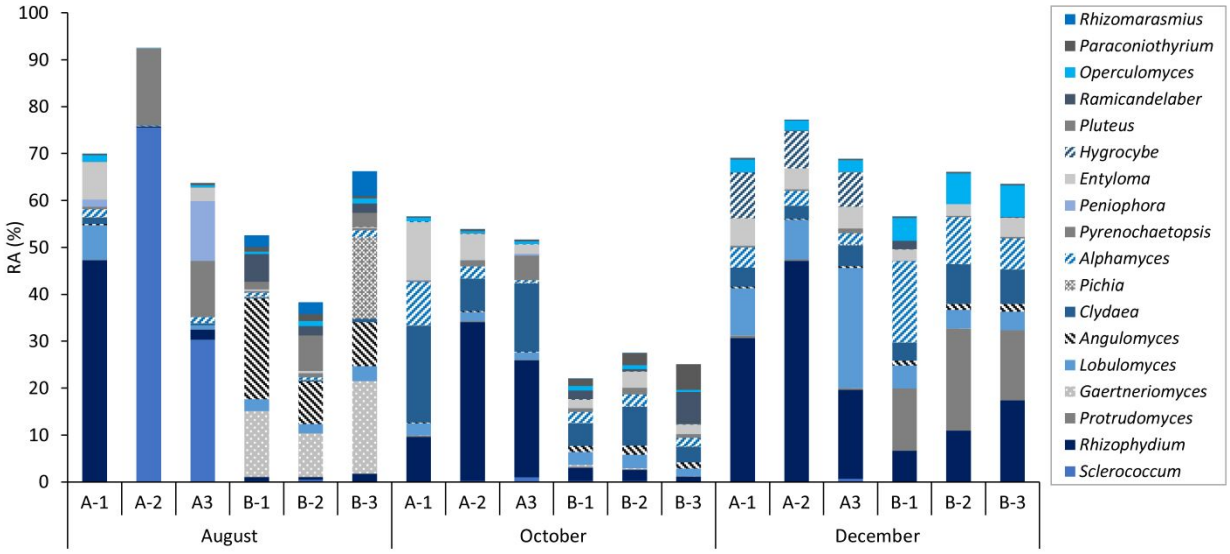

Figure 6 Relative abundance (%) of fungal communities at the genus level (all water samples) for groups with relative abundance values greater than 5% in at least one sample.

The Shannon diversity index (*H'*), sample evenness (*E*),[50] and sample richness (*R*) (determined from rarefaction curves) are shown in Figure 7. *H'*, *E*, and *R* were all markedly affected by the WWTP discharge (location A2) in August, which represented the lowest recorded values seen in the present study. This co-occurred with the previously-mentioned peaks in nitrite and phosphorus. Highest average *H'* values were calculated for Site B in October (4.19 ± 0.23), with diverse and even distribution of fungi among both dominant and minor fungal groups (Figure 6 and Figure S2, respectively). *H'* was also higher in the warmer months (August and October) as has been previously observed.[47,48] High flow conditions in December likely enhanced axial mixing in the Rio Grande and the canals. This coincided with converging *R* and *H'* values across all samples (both sites), along with increased similarities of the fungal communities overall (as seen in Figure 5).

	Sample	H'	E	Richness
August	A-1	2.59	0.43	960
	A-2	0.96	0.16	488
	A-3	3.10	0.52	846
	B-1	3.80	0.56	1335
	B-2	4.33	0.55	1299
	B-3	3.41	0.56	1252
October	A-1	3.38	0.50	1229
	A-2	3.28	0.42	1207
	A-3	3.34	0.51	1054
	B-1	4.14	0.63	1067
	B-2	4.49	0.72	1076
	B-3	3.95	0.57	1027
December	A-1	3.02	0.69	1143
	A-2	2.51	0.75	1124
	A-3	3.09	0.66	1020
	B-1	3.32	0.55	1038
	B-2	3.06	0.51	960
	B-3	3.30	0.55	1151

Figure 7 Heat map showing calculated Shannon diversity index (*H'*), evenness (*E*), and richness (*R*) values for all water samples.

3.3. Contribution of anthropogenic sources to fungal population dynamics

The sampling approach of this study was devised to assess impacts of surrounding anthropogenic sources under varying environmental conditions. Even the same source type (i.e., a WWTP) can yield distinctive changes in fungal diversity depending on various localized and environmental factors.[3,17] The three main conditions assessed in our work included: anthropogenic impacts under (1) low stream flow with low winds (August), (2) low stream flow with high winds (October), and (3) high stream flow with low winds (December). Based on these, a matrix of predicted impacts was generated (shown in Figure 2) and used for interpretation.

3.3.1. Impact of wastewater discharge on overall fungal populations

Depending on resistance of fungal groups to disinfection processes, the release of treated wastewater into open streams can potentially influence their community structure. This was explicitly observed for a range of genera at sampling locations A2 and A3. Groups with significant increases in relative abundance (greater

than 5-fold) are shown in Figure 8. Abundances of *Sclerococcum*, *Pyrenochaetopsis*, and *Paramicrosporidium* considerably increased in August at the WWTP discharge point (A2), as well as downstream (A3). Their corresponding limited increases in October and December are likely also attributable to their presence in the treated wastewater.

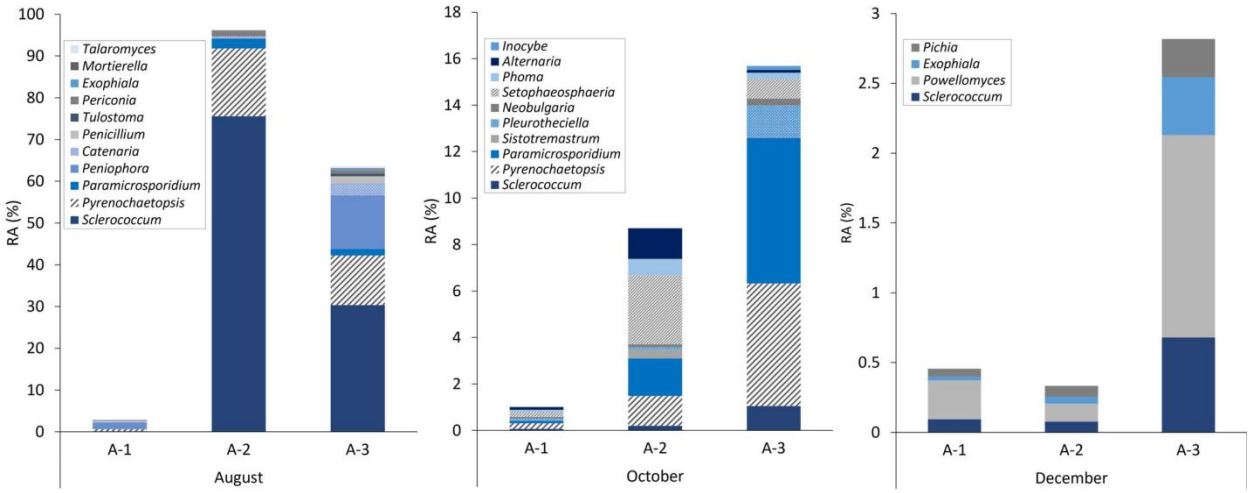

Figure 8 Relative abundance (%) of fungal communities at the genus level in water samples of Site A for groups that increased by ≥ 5-fold at the outfall (A2) and/or downstream (A3) of the WWTP discharge point.

Phoma, *Alternaria*, and *Setophaeosphaeria* were higher in A2 and A3 in October, specifically. These fungi belong to the order Pleosporales and possess the capacity to produce and release fungal spores.[51-54] *Phoma* has previously been found in various soil and water environments,[55] and *Alternaria* is known to exist in both air and water environments, including WWTPs and their surroundings.[56,57] Although the contribution of aerosols at Site A is expected to be minimal, their impact cannot be ruled out in cases of high wind speeds (i.e., October) or when wind direction is parallel to the canal (i.e., December). Still, the low occurrence of these groups in October at the upstream point of Site A (A1) is an indication that their higher abundances are more likely attributed to the wastewater effluent discharge.

At high stream flow conditions (December), the profiles of sampling points A1 (upstream) and A2 (discharge) were similar, indicating that the effects of treated wastewater release were limited. Nonetheless, a few of the less-abundant fungal groups were observed to increase at the downstream point, A3 (i.e.,

Powellomyces, *Pichia*, and *Exophiala*). Species of these genera have been detected in wastewater treatment systems and, except for the latter, are common airborne fungi.[42,58-62] Given the southern wind direction (towards A3) during the December sampling date, the increases in abundance of *Powellomyces* and *Pichia* are likely associated with the transport of bioaerosols downwind from the surface of the WWTP's activated sludge basin.

3.3.2. Effect of bioaerosols on fungal communities in adjacent surface waters

The sampling points for Site B were selected along a Rio Grande-fed irrigation canal based on their relative proximities to a WWTP (as shown in Figure 1). The downstream point (B3) was also adjacent to a livestock (dairy) facility. Both WWTPs[27,28] and dairy farms[6,7] have been previously identified as potential bioaerosol emission sources. The production and release rate of fungal spores is largely dependent on genus- or species-level variations,[63,64] with their transportation and deposition being a function of various environmental factors.[17,32] Long-distance transport is, however, limited by nutrient availability.[28]

3.3.2.1. Changes under low wind conditions

Relative abundance charts were generated to visualize the fungal groups that increased directly downstream of the WWTP (B2) and/or further downstream of the WWTP/adjacent to a livestock facility (B3) as shown in Figure 9. Several groups increased in B2 in August. Among them were *Pluteus*, *Aureobasidium* and *Exophiala*. *Pluteus* are spore-producing mushrooms of the order Agaricales with some species seen in herbivore excrement,[65] while *Aureobasidium* and *Exophiala* have been shown to occur in wastewater treatment/collection systems.[59,66,67] With the wind blowing from the SSW in August (at low speed), the higher abundance of these fungi at location B2 may have been contributed to by aerosols originating from the WWTP or the livestock facility. *Periconia* peaked in B2 in December (although at low relative abundance). This spore-producing fungus is found in water environments, commonly as aquatic saprotrophs.[68-70] With wind from the SE direction in December (away from the irrigation channels), its occurrence may have been independent of emissions from either of the facilities. *Pichia* peaked in B3 in August, with a stark increase in its relative abundance to 17%. *Pichia* is a spore-producing fungus with

some strains having been associated with cattle manure,[71] however, its co-occurrence at A3 in December suggests that it also could have originated at the WWTP.

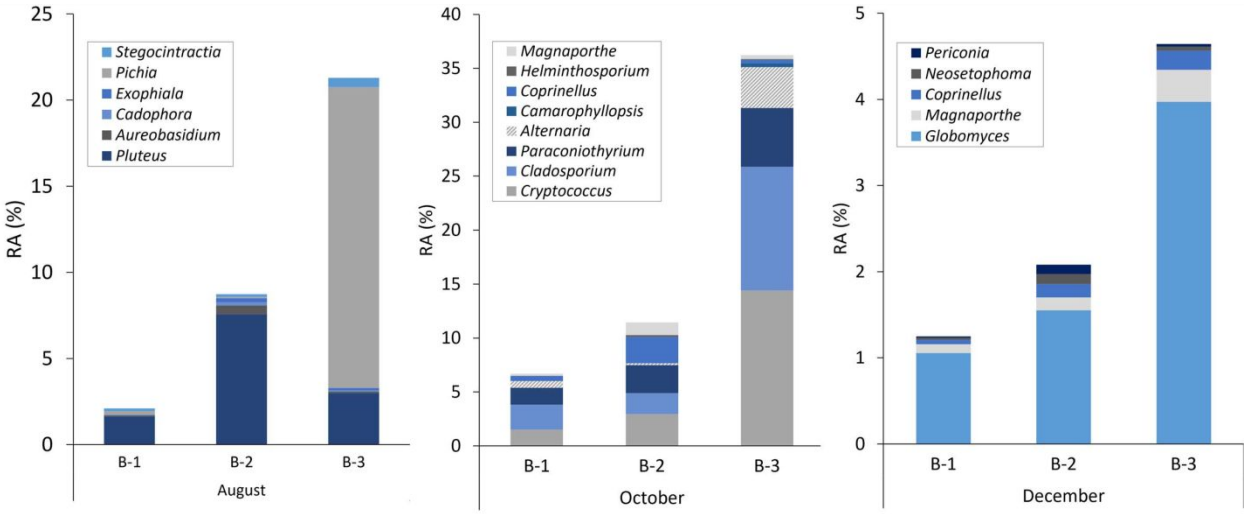

Figure 9 Relative abundance (%) of fungal communities at the genus level in water samples of Site B representing groups that increased at location B2 and/or B3 by 3-fold or greater for each of the three sampling dates.

3.3.2.2. Changes under high wind conditions

Wind speed and direction play an important role in the dispersion and transport of airborne fungi; higher winds generally facilitate broader and more distant downwind deposition of bioaerosols.[6,56] Figure 10 shows fungal groups with ≥ 5-fold higher abundances in October at Site B (when significantly higher wind speeds were observed). Several fungi were more abundant in the irrigation canal in October. Increases in *Cryptococcus*, *Cladosporium*, and *Alternaria* are mainly attributed to their aerosolization capacity and corresponding increased spread under dry high wind conditions.[72-74] For example, *Alternaria* species typically become airborne between the summer and fall seasons (i.e., August and October samples) when dry and windy weather enhance their dissemination.[75] *Ampelomyces*, falling in the order of Pleosoporales, were exclusively detected in October (Figure S4). Overall, sampling point B3 was most impacted by the high wind speeds in October. Based on the fungal groups observed to increase, this was likely a results of the combined effect of both of the adjacent anthropogenic sources.

Alternaria and *Cladosporium* release fungal spores and have previously been detected at or in areas surrounding both dairy farms and WWTPs.[56,76] *Cladosporium* is the most common airborne fungus in WWTPs as it is frequently found in activated sludge systems.[24,45] *Cryptococcus* also releases fungal spores and is widely associated with activated sludge systems.[3,17,77] Given their high abundances in B3, specifically, *Cladosporium* and *Alternaria* may also be attributable to transport from the livestock site. However, the previously documented association of *Alternaria*, *Cladosporium*, and *Cryptococcus* with WWTPs, specifically, suggests that their October spikes were likely enhanced by dispersion from the activated sludge basin surface as well.

Increases were also seen in some groups under both high and low wind conditions as compared to the Site B upstream control (B1). Specifically, the higher observed abundances of *Coprinellus* and *Magnaporthe* in B2 and B3 in both October and December (Figure 9) suggest that they were dispersed to the stream due to aerosolization from either the livestock facility or the WWTP. *Coprinellus* belongs to the order Agaricales, a group known for their capacity to degrade complex organic contaminants,[78] while *Magnaporthe* harbors species that are notorious plant pathogens.[79]

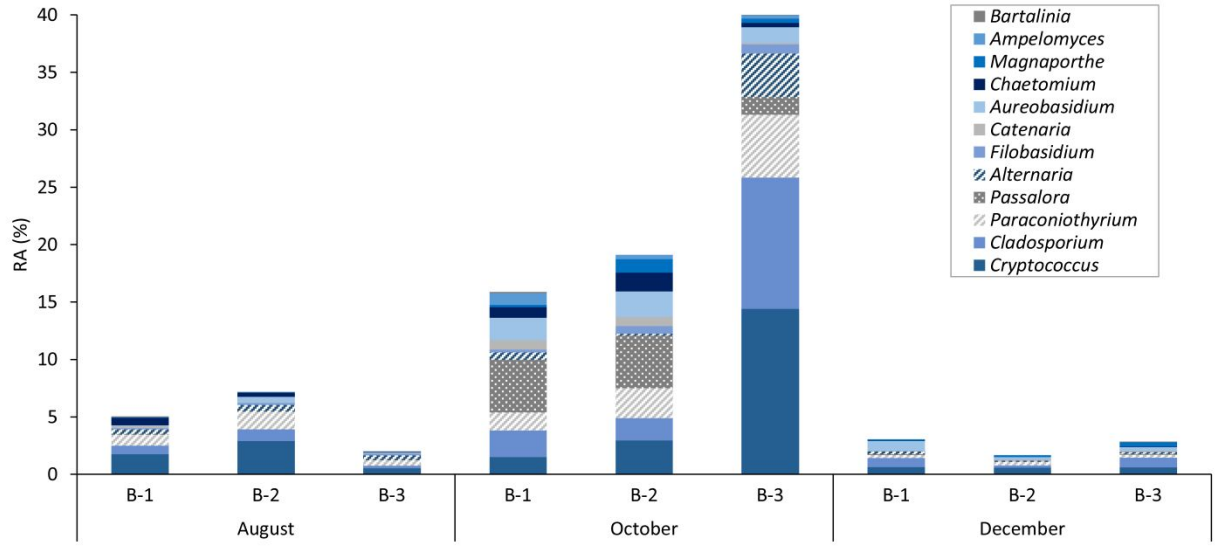

Figure 10 Relative abundance (%) of fungal communities at the genus level in water samples taken from site B and representing groups that increased during the month of October compared to August and December.

3.4. Correlations between water quality parameters and fungal populations

Fungi are versatile consumers of organic matter and nutrients in aquatic ecosystems, the availability of which directly impacts their growth and diversity.[80] In wastewater treatment processes, pH, salinity, nitrate, nitrite, and phosphorus are factors that can directly influence community structure.[4,5,17] With this in mind, we utilized Pearson correlation (*r*) and multi-linear regression (MLR) analyses to assess the relationship between fungal community dynamics and variations in water quality parameters of all samples taken.

Based on Pearson correlation, the significantly higher turbidity at Site B in August correlated with higher abundances of *Angulomyces* (r = 0.603) and *Gaertneriomyces* (r = 0.695). The drop in Shannon diversity index (*H'*) in A2 co-occurred with the peak in phosphorus concentrations (r = -0.632). Statistical analysis using MLR showed that combined variations in other parameters (specifically, nitrate and ammonia) may have also affected *H'* values. In turn, these changes directly correlated with the dominance of two distinct fungal groups, *Sclerococcum* and *Pyrenochaetopsis* (Figure 6). This is an indication that the discharge of treated wastewater – through its effect on irrigation channel nutrient concentrations – may have been partly responsible for changes in the dominant fungal groups in August.

Table 2 Pearson correlation coefficients (*r*) for correlation between tested water quality parameters and the Shannon diversity index (*H'*), total fungal abundance (using 18S rRNA gene quantitation), and relative abundances of fungal groups. The corresponding *r*-values were determined based on a two-tailed T-distribution table.

Parameter	Variable	Pearson coefficient (*r*)
Phosphorus	Shannon diversity index (*H'*)	-0.632**
Conductivity		-0.547*
Nitrate	*Protrudomyces*	0.608**
	Rhodotorula	-0.723**
	Cryptococcus	-0.645**
	Cladosporium	-0.579*
	Alternaria	-0.562*
Nitrate	Total fungal abundance	-0.575*
TS		0.649**
Ammonia	*Coralloidiomyces*	-0.791**
	Operculomyces	-0.695**
Phosphorus	*Sclerococcum*	0.799**
	Pyrenochaetopsis	0.738**
	Periconia	0.758**
TSS/TDS	*Angulomyces*	0.729**
	Gaertneriomyces	0.875**
	Rhizomarasmius	0.877**
	Boothiomyces	0.814**
	Pluteus	0.813**
Turbidity	*Angulomyces*	0.603**
	Gaertneriomyces	0.695**
	Rhizomarasmius	0.697**
	Boothiomyces	0.745**
	Pluteus	0.784**
COD	*Penicillium*	0.756**
	Aquanectria	0.635**
	Catenaria	0.832**
Conductivity	*Fusarium*	-0.624**
Turbidity		0.628**
Nitrite	*Trichoderma*	0.618**
	Periconia	0.614**
	Pyrenochaetopsis	0.652**

Significance of correlation: * indicates *P*-value < 0.05 and ** indicates *P*-value < 0.01

Other groups appeared to be mostly affected under low flow conditions. *Penicillium* was highest in A3 in August while also showing a significant correlation with COD concentrations ($r = 0.756$). This fungus is common in activated sludge systems and surface waters.[8,45] *Trichoderma* had the highest average in August (0.64 ± 0.47%) and may have also been affected by COD, nitrite, and phosphorus concentrations (Table 3). This genus has been found to be more abundant in warmer months, growing best at temperatures of 27 °C.[4,81,82] This aligns with the lower *Trichoderma* abundance in December when temperatures reached

7 °C (Table 1). *Pichia* (common in cattle environments) was highest in B3 in August and correlated with several parameters, notably the TSS/TDS ratio and nitrite concentrations. *Aquanectria*, previously shown to be higher when nutrients are more abundant,[83] was highest in A3 in August (Figure S2) while having a positive correlation with ammonia and nitrate. Abundances of *Rhodotorula*, *Cryptococcus*, *Cladosporium*, and *Alternaria* all significantly correlated with nitrate (Table 2), their high relative abundances in October, specifically, coinciding with low nitrate concentrations.

Under high flow conditions, *Candida*, *Operculomyces*, *Coralloidiomyces*, and *Protrudomyces* all saw their highest relative abundances, coinciding with changes in several parameters (Table 2). MLR showed that relative abundances of *Operculomyces* were potentially negatively affected by ammonia concentration, while *Candida* was additionally positively correlated with water conductivity (Table 3). In particular, lower overall ammonia levels in December were accompanied by the highest average abundance of *Operculomyces* (4.26 ± 1.9%), while these lower ammonia concentrations combined with higher conductivity at Site A corresponded with the highest *Candida* abundance in Site A samples in December (0.72 ± 0.12%). *Candida* species have been previously detected in WWTP environments.[47]

Table 3 Coefficients of multi-linear regression analysis showing the combined effect of several water quality parameters (independent variables) on the Shannon diversity index, total fungal abundance, and specific fungal populations (dependent variables).

Dependent variable	Independent variables	R^2	Coefficients
Shannon diversity index (*H'*)	Nitrate	0.604	-0.337
	Ammonia		-0.645
	Nitrite		0.740
	Phosphorus		-0.88**
Total fungal abundance	TS	0.688	1.86E5*
	Nitrate		-6.4E5**
	H'		-2.38E5
Cryptococcus	Nitrate	0.444	-2.43*
	H'		0.77
Rhodotorula/ *Cladosporium*	Ammonia	0.676/ 0.374	-0.156*/-0.911
	Nitrate		-0.243**/-2.101**
Penicillium	COD	0.77	0.00415**
	Ammonia		0.216
	H'		0.157
	Conductivity		-0.0026
Trichoderma	COD	0.851	0.000487
	Nitrite		0.605**
	Phosphorus		-0.408**
Pichia	Ammonia	0.601	3.641
	Nitrite		-5.93*
	Nitrate		-0.667
	TSS		-106.572
	TSS/TDS		42.830**
Operculomyces	Ammonia	0.583	-2.536**
	Conductivity		-0.0025
Sclerococcum/ *Pyrenochaetopsis*	Ammonia	0.890/ 0.814	3.548/0.368
	H'		-10.680**/-2.121*
	Phosphorus		11.212*/2.344**
	Nitrite		14.047*/4.562*
Aquanectria	Nitrate	0.575	-0.012
	Ammonia		0.140
	Nitrite		0.855
	Phosphorus		-0.597*
Candida	Nitrite	0.582	0.207
	COD		-0.00097
	TSS		2.851
	Ammonia		-0.349*
	Conductivity		0.000459*
Protrudomyces	Conductivity	0.698	-0.0169*
	Nitrate		4.764*
	COD		0.022
	Ammonia		-4.64

	Turbidity	0.022
	TSS	118.268
	TSS/TDS	-45.496

Significance of correlation: * indicates *P*-value < 0.05 and ** indicates *P*-value < 0.01

3.5. Assessment of potential fungal pathogens in irrigation canals

Public health concern associated with the presence of fungi in surface waters is on the rise; known fungal pathogens can cause a wide range of human health impacts including superficial infections (e.g. skin), allergies, and invasive infections.[84] Fungal diseases are estimated to be responsible for over 1.5 million annual deaths, with species of *Aspergillus*, *Candida*, and *Cryptococcus* being the major contributors.[85] Fungal pathogenicity can also be altered by their co-existence in the environment with other microorganisms (such as bacteria), with varying impacts on human health. Some bacteria produce compounds that enhance fungal virulence, while others can inhibit their pathogenesis by limiting fungal filamentation.[86,87] Further, the lack of effective standardized diagnoses for fungal diseases, along with a limited range of antifungal agents, has led to challenges in dealing with infections clinically.[84,85,88] This reality has only been compounded by climate change, which is broadening the regions in which opportunistic fungal pathogens occur.[13,89]

Many pathogen-associated fungal groups are naturally occurring in the environment. For instance, several *Cryptococcus* species are common in soils, bird droppings, and trees, and can also be carried by animals.[90] Other examples include *Aspergillus*, *Fusarium*, and *Penicillium* species that are common in soil, plant debris, and/or decaying vegetation.[91-93] Still, activities such as wastewater treatment and animal husbandry directly increase the potential for contamination of surface waters that are used for spray irrigation of crops. WWTPs harbor fungal pathogens that can be released with the treated wastewater or aerosolized and deposited to nearby settings.[3,4,27,28] Dairy farms have also been shown to foster certain pathogens.[6, 7] These contributors can increase the incidence of waterborne fungal pathogen deposition on crops and/or inhalation of fungal spores by farmers and nearby communities. Based on this, a targeted

assessment of potential fungal pathogens was conducted as part of this study to elucidate such anthropogenic contributions.

Figure 11 represents potential pathogens that were detected in all samples, with several groups showing increases in relative abundance near or downstream of their respective contamination source. It should be noted that, because this analysis was based on genus-level classification, any observed occurrence of a potentially pathogenic group does not definitively indicate that it includes species known to cause disease. Filamentous genera such as *Aspergillus*, *Trichoderma*, *Fusarium*, and *Penicillium* harbor pathogens that are commonly detected in WWTPs and capable of mycotoxin production; all of these were detected near the WWTPs of both sites (Figure S5).[3,8,94] *Aspergillus, Penicillium*, and *Fusarium* have also been observed in the atmosphere of dairy milk farms.[6] Although occurring at low relative abundances, their high aerosolization capacity is particularly concerning for spray irrigation practices. *Aspergillus* was highest in B2 in August and October, possibly due to aerosol dispersion from the dairy facility (winds blowing from the SSW of the lot). *Aspergillus* spp. can cause invasive aspergillosis, in some cases with up to a 50% mortality rate.[36,95] *Penicillium* was highest in A3 in August (500 m downstream of the WWTP discharge) with some species known to cause invasive pulmonary mycosis and other cutaneous infections.[96,97] *Fusarium* and *Trichoderma* peaked in B2 in August, also downwind of the livestock facility. They are respectively responsible for fusariosis and other invasive fungal diseases typically targeting the immunocompromised.[98,99]

Exophiala and *Candida* were most abundant in A3 in December. *Exophiala* can cause pulmonary infection and other cutaneous infections.[100] Some *Candida* species recognized as human pathogens commonly cause candidiasis and candidemia, often in patients with weakened immune systems.[22] Chlamydospore formation of *Candida* species allows for potential transport under specific wind conditions, which has been shown to cause health concerns for farmers.[101] *Pichia* peaked in B3 in August; some of its species cause sporadic human infections (including pneumonia, fungemia, and mucosal infections), most notably in immunocompromised patients and infants in neonatal intensive care units.[102]

High winds in October were accompanied by an increase in some potential pathogens. *Rhodotorula*, belonging to the family Sporidiobolaceae, was highest overall in October Site A samples (Figure S5). Multiple *Rhodotorula* species are spore-producing human pathogens, often causing fungemia in susceptible patients with underlying diseases (e.g., cancer, AIDS, heart diseases).[5,103] Several other genera seemed to spike in B3, specifically. Among them was *Cladosporium*, a common airborne fungus often encountered in both WWTPs and livestock facilities.[5-7,17] As human pathogens, *Cladosporium* spp. are linked with allergic rhinitis and respiratory arrest in asthmatic patients.[104,105] Some *Alternaria* species are emerging human pathogens as well due to their mycotoxin production capacity. Alternariosis infections are attributed to spore inhalation (possible during spray irrigation) or consumption of contaminated crops.[75,106] Species of *Paraconiothyrium* are also common pathogens responsible for cutaneous phaeohyphomycosis in immunodeficient patients.[74,107] *Cryptococcus*, one of the most concerning fungal threats to human health, was notably higher in all samples in October, with a specific peak in B3. Inhalation of stress-resistant fungal spores of some species leads to cryptococcosis in immunocompromised individuals.[77] Infections associated with *Cryptococcus* exceed 220,000 cases annually and are responsible for nearly 15% of AIDS-associated deaths.[95,108]

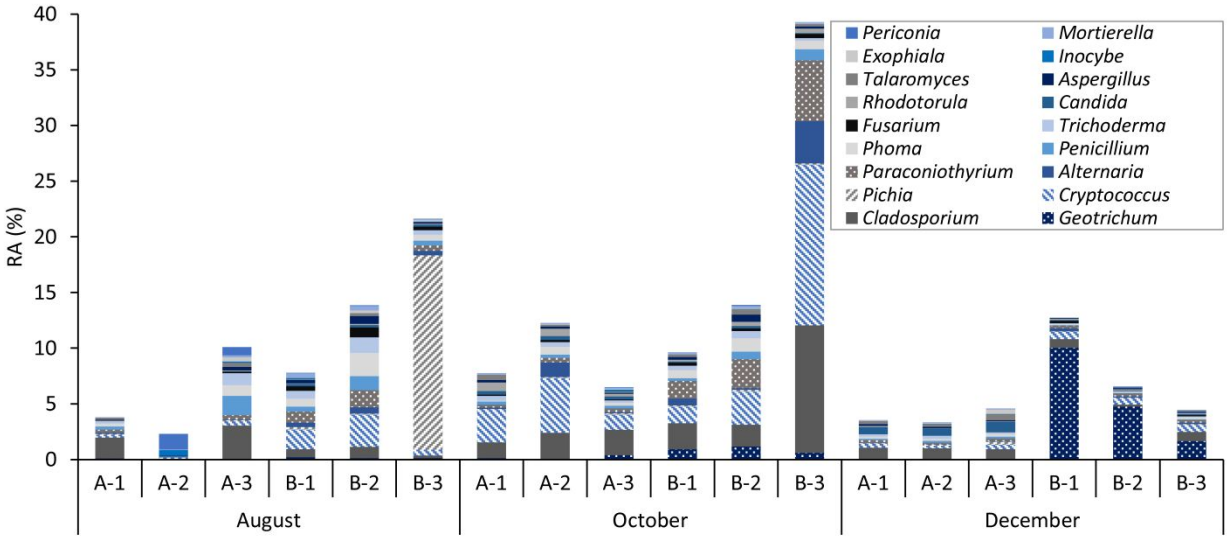

Figure 11 Relative abundance (%) of fungal communities at the genus-level representing potentially pathogenic fungi in all water samples.

This study showed a major increase in potential waterborne fungal pathogens during windy conditions, particularly with regards to *Cladosporium*, *Cryptococcus*, *Alternaria*, and *Paraconiothyrium*. Further, the higher downstream presence at Site B indicates that aerosolization from WWTPs and/or livestock operations can increase contamination in waters used for crop irrigation. This can directly impact disease occurrence among farmers (and others in nearby communities) through inhalation of associated fungal spores. It is also worth noting that the month of August – with its higher temperatures and lower relative humidity – seemed to facilitate the waterborne occurrence of several potential pathogens with high aerosolization capacity (e.g. *Penicillium*, *Trichoderma, Pichia*), accentuating the seasonality of their occurrence. High flow/low temperature conditions (December) co-occurred with the lowest average potential pathogen relative abundances, the only one with notable abundance being *Geotrichum*.

Overall, the present work provides important insight into the varying effects that bioaerosols and treated wastewater discharges can have on fungal communities in adjacent/receiving surface waters. To build upon these observations, future work would benefit from the parallel investigation of fungal occurrence within treatment systems, their discharges, and their associated receiving waterbodies. In order to expand knowledge on public health impacts, another key aspect of future investigations will be the incorporation of molecular- and culture-based approaches for species-level fungal community identification in order to definitively gauge the prevalence of pathogens in such environments.

4. Conclusion

Fungal populations are an essential component of aquatic environments. While some have key functional roles, others can be highly burdensome to human health. Rivers and their associated irrigation canals are critical water resources in regions such as the southwestern United States. Climate change is inducing increased water scarcity in such regions, but it is also giving rise to other unintended consequences such as emerging microbial threats (e.g., fungal pathogens). In this context, irrigation canals harbor intensified potential for exposure to fungal contamination from anthropogenic sources such as WWTPs. Occurrence of these emerging pathogens can be amplified by the discharge of treated effluents (received by the canals)

or through bioaerosolization and dispersal from open-air treatment basins. Taking this into consideration, locations were selected to evaluate the impact of treated wastewater discharge (Site A) and aerosol emissions (Site B) on the irrigation canal fungal communities. Results showed that both scenarios had notable effects on fungal community structures in adjacent irrigation canals. Samples taken at Site A were directly influenced by the effluent discharge from a WWTP under low flow/low wind conditions in the month of August. Effects of bioaerosol emissions (Site B) were notable across all sampling dates, but were amplified under low flow/high wind conditions (October). Limited variations in fungal communities and the lowest abundances of potential pathogens were observed under high flow/low wind conditions in December. The elevated abundances of potential pathogens observed during high winds (including *Alternaria*, *Cladosporium,* and *Cryptococcus*) accentuate the need to broaden our knowledge on anthropogenic contributions to waterborne fungal occurrence and dispersal.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

All high-throughput sequencing files used for analysis are publicly available on the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under study accession number PRJNA1208059. All other data generated and analyzed during this study are included in this article or its supplementary information file.

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Appendix A: Supplementary data

Supplementary information to this article is provided in Appendix A and includes: details of ITS MiSeq amplicon sequencing, the methodology for growth of *Saccharomyces cerevisiae* culture, DNA isolation, qPCR amplification details, PCR primers used for ITS amplicon sequencing (Table S1), qPCR primers used for 18S rRNA gene quantification and corresponding thermocycling conditions (Table S2), Pearson correlation coefficients between water quality parameters (Table S3), conductivity and turbidity values of water samples (Figure S1), relative abundance (%) of fungal communities at the genus-level for water samples with relative abundances > 1% (Figure S2), 18S gene copy numbers for water samples (Figure S3), relative abundances of fungal communities in water samples from Site B showing an increase in October with relative abundances < 1.5% (Figure S4), and relative abundances of fungal communities representing potentially pathogenic fungi in all water samples with relative abundances < 2% (Figure S5).

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Data availability

All high-throughput sequencing files used for analysis are publicly available on the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under study accession number PRJNA1208059. All other data generated and analyzed during this study are included in this article or its supplementary information file.