Appendix A: Supplementary information

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

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ITS MiSeq amplicon sequencing methods:

Amplicon sequencing was performed at the MR DNA Lab (www.mrdnalab.com, Shallowater, TX, USA) using the MiSeq platform. DNA template was first amplified using PCR and the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with the primer set ITS1F/ITS2 (Table S1). Thermocycling conditions were as follows: 95 °C for 5 min followed by 30 cycles of 95 °C for 30s, 53 °C for 40s, 72 °C for 60s, and a final extension at 72 °C for 10 min. The PCR product was then run on a 2% agarose gel for amplification confirmation. Samples were subsequently multiplexed, pooled, then purified using calibrated Ampure XP beads, and finally used to prepare the corresponding DNA library. Generated data as fastq files were processed and analyzed using MR DNA analysis platform (MR DNA, USA)

Table S1 Primer sequences used for ITS amplicon sequencing

Gene	Primers (5'-3')	Amplicon (bp)	Reference
ITS1f/ ITS2	F- CTTGGTCATTTAGAGGAAGTAA R- GCTGCGTTCTTCATCGATGC	250	(Bokulich & Mills, 2013)

Growth of *Saccharomyces cerevisiae* culture and DNA isolation:

Saccharomyces cerevisiae species was cultured and grown in the lab in small volumes on a Sabouraud Dextrose Agar (SDA) medium according to the manufacturer's guidelines (Ward's Science, USA). To prepare 1L of SDA medium, we added 40 g of glucose, 10 g of peptone, and 20 g of agar powder, with pH adjusted to 5.6. The content was mixed and then autoclaved at 121 °C for 15 mins. After allowing it to cool to 60 °C, the medium was poured into 60 mm sterile petri dishes and allowed to solidify at room temperature for at least 30 minutes. Meanwhile, the tube of *Saccharomyces cerevisiae* with a slightly loose cap was placed inside a beaker filled with water to cover the level of medium inside the tube. The beaker was heated on a hot plate and boiled for a few minutes to allow for the liquefaction of the medium inside the tube. Once the content melted, heat was turned off and it was left to cool down to 45-50 °C. The mouth of the tube was flamed to minimize contamination. Contents were plated and incubated at 30 °C for 5-7 days, after which colonies were inoculated and grown for another 5 days in 5 mL of SD liquid medium (consisting of SDA without agar powder). 2 mL of media with the grown culture was centrifuged at 12,000 rpm for 10 mins, the supernatant discarded, and the pellet used for subsequent DNA extraction.

Given the variable nature of the ITS-sub-regions, it can increase potential bias during quantification by over-amplification of certain taxonomic groups (Bellemain et al., 2010; Nilsson et al., 2009). As an alternative, the 18S rRNA gene was used for quantification of total fungal abundance in the environmental samples (Evans & Seviour, 2012; Maza-Márquez et al., 2018; Zhang et al., 2015). The extracted DNA template from the culture was amplified by PCR using the selected 18S rRNA gene primers (Table S2) with the following conditions: 95 °C for 2 min followed by 95 °C for 10 s, 60 °C for 40 s, 72 °C for 30s, and a final extension at 72 °C for 7 min. The PCR product was run on a 1.5% agarose gel at 100 V for 25 mins. The resulting band was excised and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Concentrations were measured on a Quantus fluorometer (Promega, USA) to determine the corresponding copy number for the gene of interest (18S). Serial dilutions from 10^{-1} to 10^{-8} (corresponding to a known copy number) were prepared from the purified PCR product of the *Saccharomyces cerevisiae* and used to generate the standard curve of the 18S rRNA gene. An amplification efficiency of 99% and linear relationship of 0.998 were achieved. The curve was then utilized to determine the copy number of each sample based on obtained cycle threshold (Ct) values. Amplicon and primer specificities was verified using melt curve analysis by increasing the temperature from 60 °C to 95 °C at 1 °C increments.

Table S2 Primer sequences, thermocycling conditions and amplicon size for 18S rRNA gene used for qPCR amplification

Gene	Primers (5'-3')	Pre-incubation	Amplification	Cycles	Amplicon (bp)	Reference
18S	F- GTAGTCATATGCTTGTCTC R- ATTCCCCGTTACCCGTTG	95°C for 2 min	95°C for 30s, 60°C for 40s, 72°C for 30s	40	337	(Zhang et al., 2015)

Table S3 Matrix representing the Pearson correlation coefficients (*r*) between the different tested water quality parameters. Cells highlighted in red indicate a strong negative correlation and cells highlighted in green indicate a strong positive correlation.

	Nitrate	*COD*	*Ammonia*	*Nitrite*	*Conductivity*	*turbidity*	*TS*	*TSS/TDS*
Nitrate	1							
COD	-0.615*	1						
Ammonia	-0.376	0.318	1					
Nitrite	-0.326	0.485	0.740*	1				
Conductivity	-0.117	0.274	-0.102	0.025	1			
turbidity	-0.373	0.014	0.566*	0.272	-0.106	1		
TS	-0.211	0.129	-0.091	-0.148	0.758*	0.113	1	
TSS/TDS	-0.299	-0.039	0.597	0.251	-0.323	0.897*	-0.166	1

* indicates p-value < 0.05

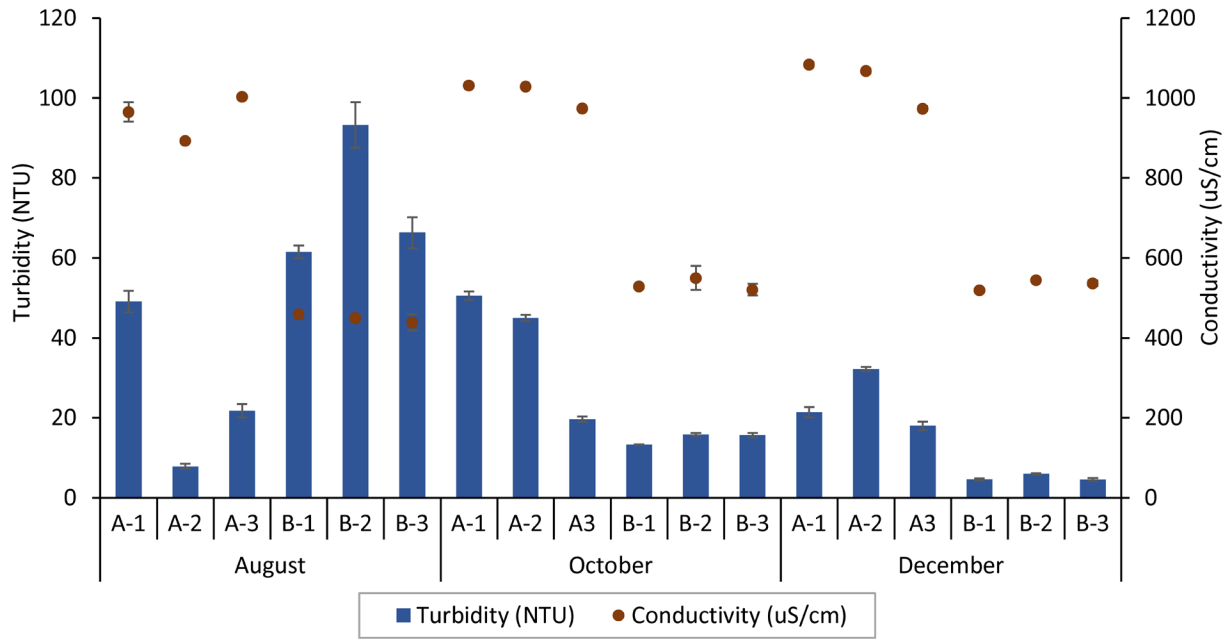

Figure S1 Conductivity and turbidity values measured for all water samples collected in August, October, and December.

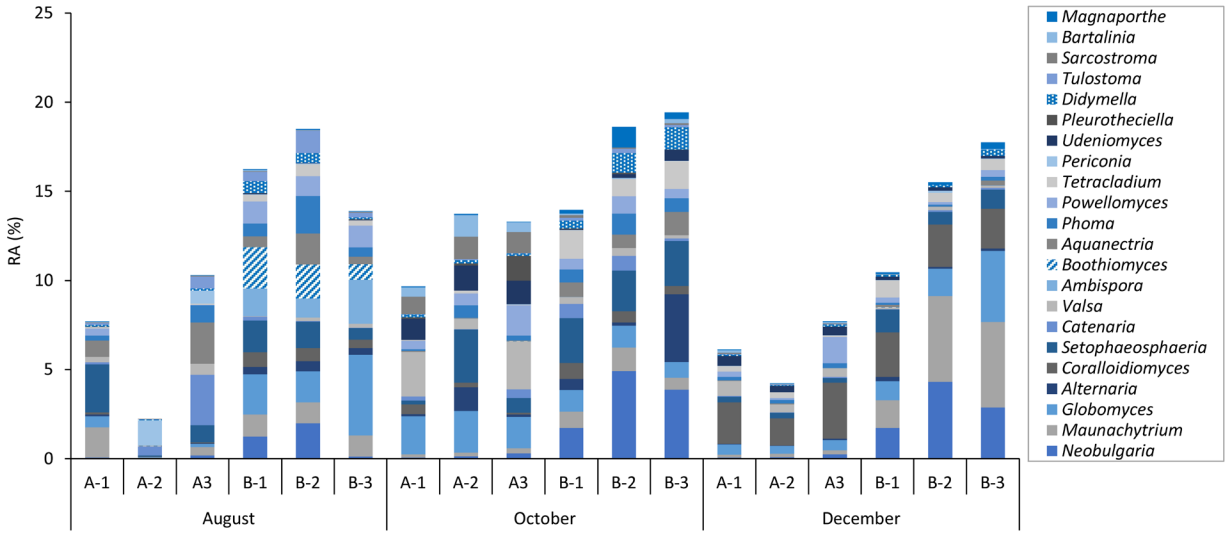

Figure S2 Relative abundance (%) of fungal communities at the genus level for all water samples with relative abundance values greater than 1%.

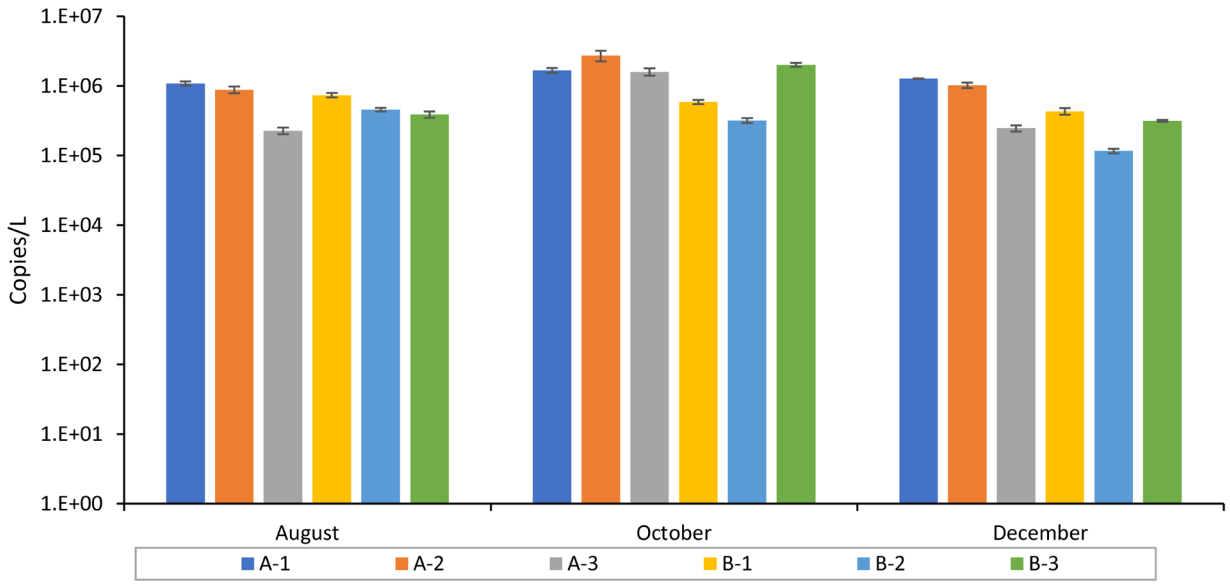

Figure S3 18S gene copy number representing the total fungal abundance of all water samples collected from the irrigation canals during the months of August, October, and December.

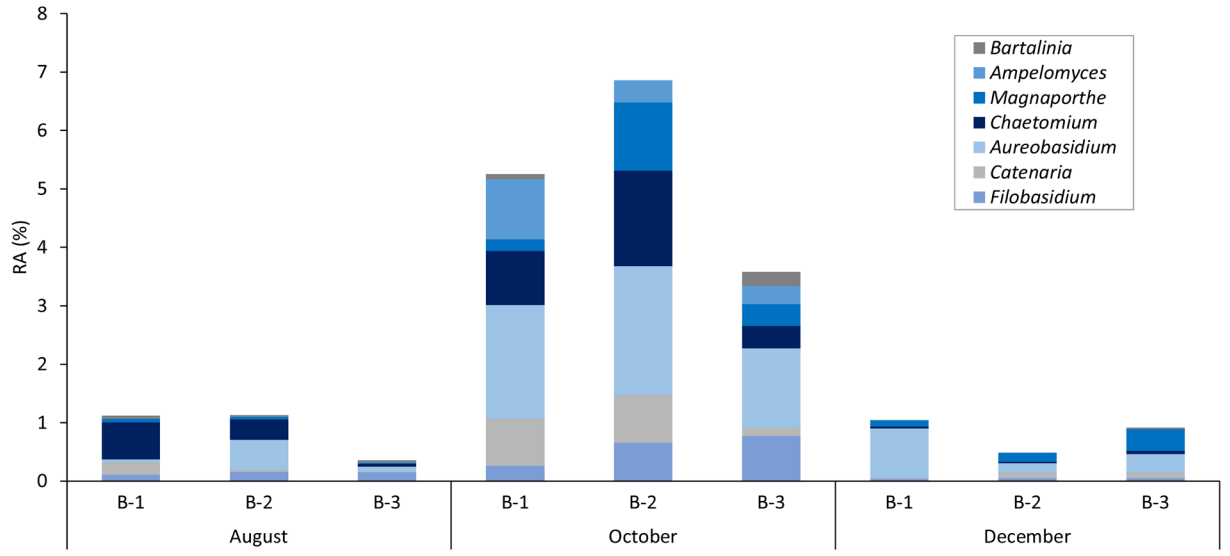

Figure S4 Relative abundance (%) of fungal communities at the genus level in water samples taken from site B and representing groups that had more than a 5-fold increase during the month of October compared to August and December, and having relative abundance <1.5%.

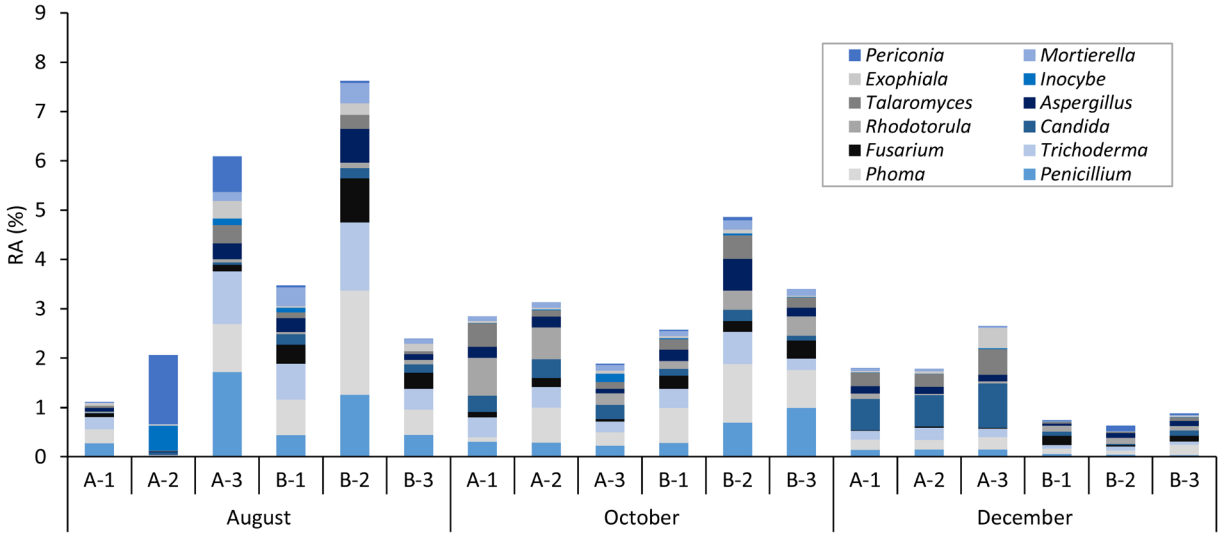

Figure S5 Relative abundance (%) of fungal communities at the genus-level representing potentially pathogenic fungi in all water samples with relative abundance <2%.

References

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