Case Report: Contamination of a Drinking Water Distribution System by *Exophiala*-dominated Biofilm in the Midwestern United States

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Abstract

Fungal contamination of drinking water distribution systems can impact water quality with implications for public health. We document several *Exophiala* spp. biofilm contamination events at customer taps in the Midwest United States (Ohio) following consumer complaints. Three samples of biofilm were collected and processed using next-generation DNA sequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer region. Two samples with successful fungal sequencing were dominated by *Exophiala* spp., putatively identified as *E. cancerae*, *E. lecanii-corni*, and *E. oligosperma*. The dominant bacterial phyla were Proteobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria. Bacterial composition varied substantially at the family and genus levels. Presence of potentially pathogenic bacteria (i.e., *Acinetobacter* spp., *Legionella* spp., *Mycobacterium* spp., and *Pseudomonas* spp.) and fungi (i.e., *Exophiala* spp., *Knufia spp.*, *Cyphellophora* spp., *Ochroconis* spp., *Rhinocladiella* spp.) suggests these biofilms could be of public health concern.

Keywords: bacteria; faucet; fungi; microbial communities; opportunistic pathogens; shower head

Introduction

Contamination of drinking water distribution systems by microorganisms has been recognized since the mid-1800s, and contamination events may result from introduction and/or regrowth of bacteria, viruses, protozoa, and fungi (Rochelle and Clancey 2006). For example, contamination with opportunistic pathogen bacteria such as *Acinetobacter baumannii*, *Legionella pneumophila*, and *Mycobacterium avium* is well-known (Falkinham 2011; Carvalheira et al.

2021; CDC 2021) with healthcare costs from these three species estimated at $600 million annually for the elderly in the United States (Naumova et al. 2016).

Fungal contamination of drinking water distribution systems is less frequently studied but is increasingly recognized (Mhlongo et al. 2019) with impacts upon water quality (e.g., color, odor, and taste), degradation of materials, and concerns about mycotoxin exposure and opportunistic infections (Nucci et al. 2002; Hageskal et al. 2009; Mesquita-Rocha et al. 2013; Mhlongo et al. 2020; Afonso et al. 2021). Available reports of fungal growth within distribution systems primarily implicate common, terrestrial, and filamentous genera, including *Aspergillus*, *Cladosporium*, and *Penicillium* (Afonso et al. 2021). These may co-occur with bacteria and protozoa in biofilm communities, and interkingdom interactions within such biofilms are poorly understood (Afonso et al. 2021).

Aside from common terrestrial fungi, members of the black yeast genus *Exophiala* are occasionally reported as distribution system contaminants in tap water and especially around outlets in bathrooms, kitchens, dishwashers, and laundry machines (Matos et al. 2002; Lian and De Hoog 2010; Adams et al. 2013; Isola et al. 2013; Biedunkiewicz and Schulz 2012; Babič et al. 2016; Moat et al. 2016; Zupančič et al. 2016; Babič et al. 2017; Wang et al. 2018; Kulesza et al. 2021). Within such environments, oligotrophy and tolerance of extreme conditions by certain *Exophiala* species enables their growth (Hamada and Abe 2010; Lian and De Hoog, 2010; Heinrichs et al. 2013b; Zupančič et al., 2016; Wang et al. 2018; Kulesza et al. 2021; Romsdahl et al. 2021). Moreover, many *Exophiala* spp. are opportunistic pathogens affecting both immune-competent and immune-compromised persons (Zeng et al. 2007; Sav et al. 2016; Singh et al. 2021; Usuda et al. 2021). Infections with *Exophiala* spp. are most often superficial but do include deep-tissue and systemic mycoses which most commonly affect the lungs (Zeng et al.

2007; Woo et al. 2013; Usuda et al. 2021). Dermal contact, ingestion, and inhalation may be relevant routes of exposure.

Recently, Heinrichs et al. (2013a, b) investigated black biofilms growing on aerators, shower heads, and toilet tanks in Germany. These biofilms were dominated by *Exophiala lecanii-corni* and smaller amounts of other *Exophiala* spp and black yeast-like fungi. *E. lecanii-corni* may cause superficial mycoses effecting skin, nails, eyes, and sinuses in addition to deeper mycoses of the lungs, digestive system, and central nervous system (Futatsuya et al., 2023; Hatta et al., 2021; Lee et al., 2016; Miyakubo et al., 2020; Woo et al., 2013; Zeng et al., 2007) . After further sampling of that distribution system, retrograde contamination with *E. lecanii-corni* was suggested (Heinrichs et al. 2013b). However, it is unknown how frequently similar, extensive *E. lecanii-corni* biofilms contaminate other distribution systems.

In this study, we report a series of *Exophiala* spp. biofilm contamination events at taps within a central Ohio (USA) distribution system similar to that reported by Heinrichs et al. (2013a). Our objective was to characterize these biofilms through DNA sequencing of the bacterial 16S and fungal ITS regions and to identify potentially pathogenic taxa of concern to water resource managers and for public health. This work highlights the potential importance of fungal biofilms in drinking water systems.

Methods

Three biofilm samples were collected during November 2022 from homes that belong to a central Ohio, USA distribution system (Figure 1). Samples were collected from an area within the distribution system where multiple homeowners had complained to operators about excessive

biofilm growth on taps. Biofilms growing on kitchen sinks (i.e., samples S1 and S2) and a shower head (i.e., sample S3) were collected without prior flushing, using sterile cotton swabs and 4 oz Whirl-Pak® bags (Pleasant Prairie, WI, USA). Samples were promptly transported to The Ohio State University and stored at -20 °C. Microscopic observation, DNA extraction procedure, Illumina sequencing, and bioinformatics are detailed in supplemental materials.

Results and Discussion

Fungal sequences were identified for samples S1 and S2, which yielded 36,342 and 26,873 sequences per sample respectively, before denoising. Sample S3 failed to amplify during ITS sequencing. Both samples were dominated Order Chaetothryiales, and specifically by *Exophiala* spp. (Table 1). In sample S1, the putative species *E. cancerae* (85% of the reads) and *Knufia epidermidis* (11% of the reads) were dominant, whereas in S2, the putative species *E. lecanii-corni* was dominant (98% of the reads). *E. lecanii-corni* dominated the biofilm samples characterized by Heinrichs et al. (2013a). We view the identification of *E. cancerae* with caution because species-level identifications from next-generation DNA sequencing are tentative owing in part to sequencing and database shortcomings (Nilsson et al. 2006; Yamamoto et al. 2014). Moreover, *E. cancerae* is primarily reported from tropical locations. In South America, it is a causative agent of Lethargic Crab Disease (Orélis-Ribeiro et al. 2011) and we are aware of one report of gastrointestinal infection by *E. cancerae* from Hong Kong (Woo et al. 2013).

Several additional melanistic, black yeast-like fungi from orders Chaetothryiales and Venturiales that are commonly found in bathrooms (Lian and de Hoog 2010; Wang et al. 2018), and that are capable of human opportunism were detected. First, *E. oligosperma* (0.6% of reads

in S2) opportunistically infects cutaneous, subcutaneous, and various deep tissues including the lungs, heart, gastrointestinal tract, spleen, lymphatic system, blood, and brain (Tintelnot et al. 1991; de Hoog et al. 2003; al-Obaid et al. 2006; Zeng et al. 2007; Woo et al. 2013). Several additional species that opportunistically primarily infect human skin and nails were also detected, including *Knufia epidermidis* (11% of reads in S1; Li et al. 2008; Saunte et al. 2012; Martin-Gomez et al. 2019), *Cyphellophora europaea* (4% of reads in S2; de Hoog et al. 2000; Lian and de Hoog 2010; Saunte et al. 2012; Feng et al. 2014), *Rhinocladiella similis* (<0.001% of reads in S2; Lian an de Hoog 2010; Richarz et al. 2018; de Hoog et al. 2003), and *Ochroconis mirabilis* (0.1% of reads in S1; Giraldo et al. 2014; Shi et al. 2016; Yew et al. 2016).

Bacterial sequencing was successful for all samples with 25,019 to 44,339 sequences per sample before denoising. Across all samples, 114 amplicon sequence variants (ASVs) were identified. Only 19 ASVs (17%) were detected in all three samples and 31 additional ASVs (27%) were present in two samples. Measures of alpha diversity after rarefaction were computed, including Shannon Entropy (Shannon 1948) and Chao 1 Index (Chao 1984) (Figure 2). Shannon diversity values were comparable to previous analyses of biofilms within water distribution systems (Gomez-Smith et al. 2015; Ren et al. 2024), whereas Chao I values were lower (Cruz et al. 2020).

Four phyla – Proteobacteria, Bacteroidetes, Acidobacteria, and Actinobacteria – were present in all samples, accounting for 70-97% of reads (Figure 3). Bacterial composition of samples was similar at the phylum and class levels, with more differentiation at the family and genus levels (Figure 3) as reported previously (Li et al. 2016). Across different geographic regions and distribution system designs, predominant phyla in distribution system biofilms are Proteobacteria, Actinobacteria, Acidobacteria, Cyanobacteria, Bacteroidota, Nitrospira,

Firmicutes, and Planctomycetota (Proctor and Hammes 2015; Li et al. 2016; Stanish et al. 2016; Cruz et al. 2020; Ren et al. 2024). The most abundant classes identified in our samples, *Alphaproteobacteria, Betaproteobacteria, Cytophagia* and *Gammaproteobacteria*, were also detected in a German distribution system, where biofilm samples also displayed high community variance (Henne et al. 2012). The possible opportunistic pathogens *Legionella* spp., *Pseudomonas* spp., *Mycobacterium* spp., and *Acinetobacter* spp. were all detected in at least one sample, as in previous studies (Douterelo et al. 2014; Li et al. 2016; Waak et al. 2018). Certain members of these genera are capable of growth within distribution system biofilms, resulting in illness (Falkinham 2011; Waak et al. 2018; Carvalheira et al. 2021). Moreover, emerging evidence suggests microbial communities in drinking water influence human health through the microbiome (Bowyer et al. 2020; Lugli et al. 2022; Vanhaecke et al. 2022). Microbiome impacts from ingesting the bacterial and fungal communities we describe are unknown.

Beyond health implications, identification of ecological processes promoting growth of biofilms dominated by *Exophiala* and other black yeast-like fungi may assist control efforts. *E. lecanii-corni* is resistant to temperature, osmotic, and oxidative stresses (Romsdahl et al. 2021), is oligotrophic and exhibits extreme shear strength (Heinrichs et al. 2013b), and thrives in environments laden with toxic hydrocarbons (Woertz et al. 2001; Pirnie-Fisker and Woertz 2007). For these reasons, Heinrichs et al. (2013b) proposed that VOCs from cosmetics or cleaning may contribute to biofilm contamination. Other considerations for future studies include depletion of chlorine residual, microbial regrowth and its promoting conditions, and water age. In the distribution system sampled, contamination events were somewhat clustered, especially in areas where construction activity necessitated reduction of flow for extended periods. Future

158 studies of these biofilms could sample distribution systems more extensively and seek to
159 understand the source and conditions that encourage growth.
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162 **Conclusions**
163 We document occurrence of *Exophiala*-dominant biofilm on distribution system taps
164 following Heinrichs et al. (2013a, b), this time in the Midwestern USA. Additionally, we report
165 on the bacterial composition of these biofilms. Biofilms samples contained potentially
166 pathogenic bacteria and fungi including *Acinetobacter* spp., *Legionella* spp., *Mycobacterium*
167 spp., *Pseudomonas* spp., *Exophiala* spp., and *Knufia* spp. Health implications of these biofilms
168 are uncertain. Future studies might include more extensive sampling of drinking water
169 distribution systems for fungal contamination and identifying the environmental conditions that
170 support growth to inform future control efforts.
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172
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179
180 **Data Availability**

Raw sequences are available from GenBank (BioProject: PRJNA1072827).

Conflict of Interest

The authors have no conflicts of interest to declare.

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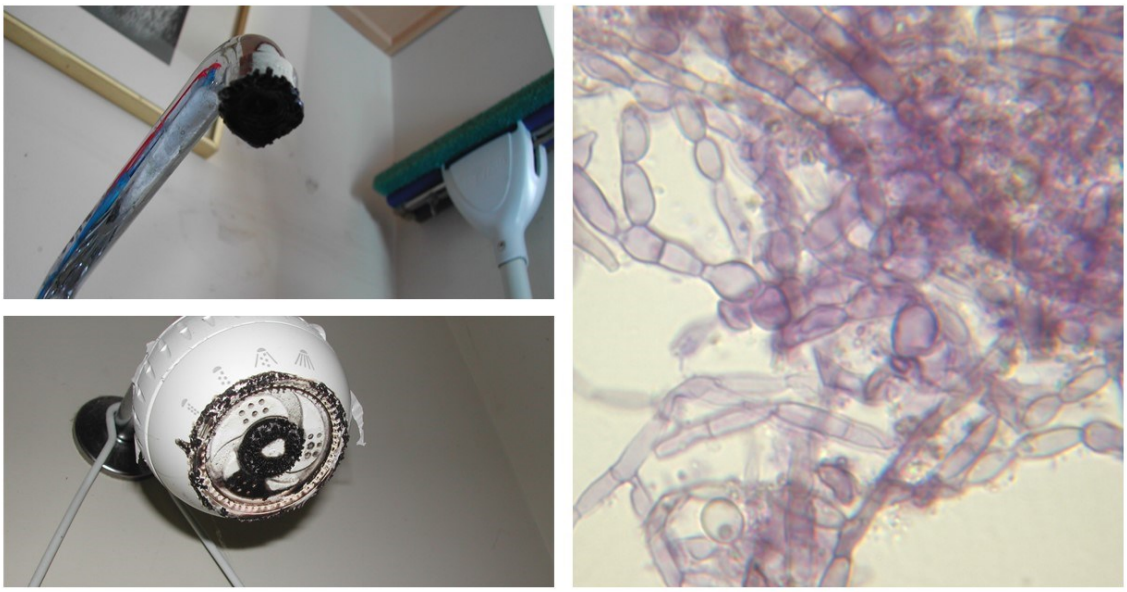
Tables

Table 1. Read counts of putative fungal species identified through ITS sequencing.

Species	S1	S2
Exophiala cancerae	20196	0
Exophiala lecanii-corni	834	14447
Knufia epidermidis	2574	48
Fusarium acutatum	16	87
Exophiala oligosperma	0	95
Dactylella zhongdianensis	84	0
Cyphellophora europaea	0	65
Ochroconis mirabilis	30	0
Cyphellophora reptans	0	31
Cyphellophora guyanensis	0	6
Metacordyceps chlamydosporia	0	12
Cystobasidium slooffiae	1	0
Schizothecium inaequale	1	0
Naganishia albida	0	1
Rhinocladiella similis	0	1
Species unknown	0	1

Figures

Figure 1. Biofilms on customer taps (left) and light microscope image of biofilm stained with crystal violet solution at 1000× magnification (right).



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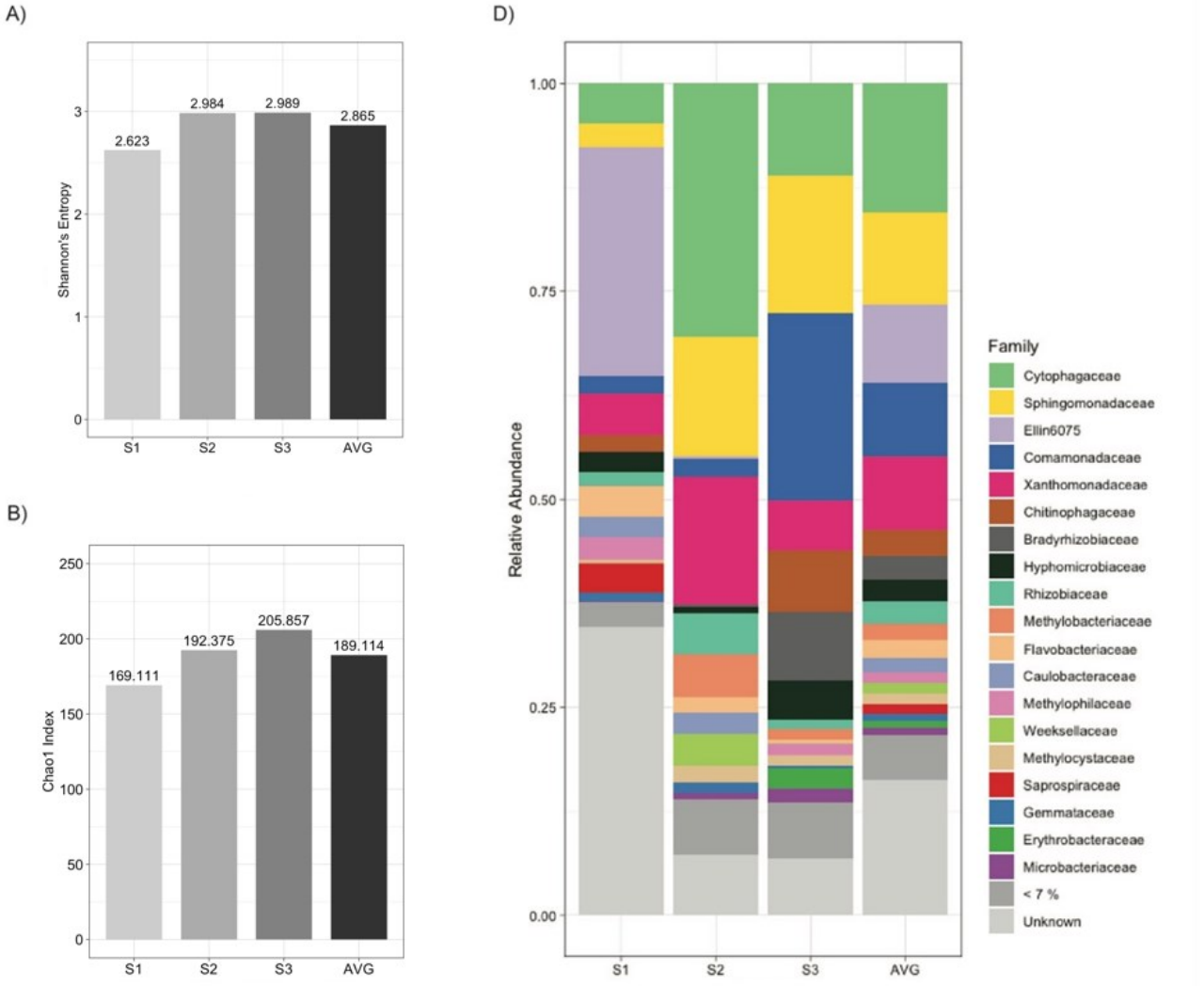
430 **Figure 3.** Summary of bacterial communities in biofilm samples including A) Shannon index, B)

431 Chao 1 index, C) the top five most abundant taxa at phylum, class, family, and genus ranks, and

432 D) relative abundance of bacterial families.



C)

Phyla	RA	Class	RA	Family	RA	Genus	RA
Proteobacteria	0.51	Alphaproteobacteria	0.30	*Cytophagaceae*	0.15	*Spirosoma*	0.10
Bacteroidetes	0.24	Cytophagia	0.16	*Sphingomonadaceae*	0.11	*Pseudoxanthomonas*	0.06
Acidobacteria	0.09	Betaproteobacteria	0.11	*Ellin6075*	0.09	*Sphingopyxis*	0.05
Actinobacteria	0.02	Gammaproteobacteria	0.10	*Comamonadaceae*	0.09	*Sphingobium*	0.03
Cyanobacteria	0.02	Chloracidobacteria	0.09	*Xanthomonadaceae*	0.09	*Hyphomicrobium*	0.02

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