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Characterization of Fungal Biofilms within a Municipal Water Distribution System

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Biofilms of a municipal water distribution system were characterized to assess the occurrence of fungi within surface matrixes. Densities of filamentous fungi ranged from 4.0 to 25.2 CFU cm^{-2}, whereas yeast densities ranged from 0 to 8.9 CFU cm^{-2}. Observations by scanning electron microscopy further suggested that spores, not hyphae or vegetative cells, comprised the primary source of viable propagules.

The presence of fungi in drinking water and within biofilms of distribution systems has received limited attention. This is due, in part, to the fact that causal relationships between fungal occurrence and water quality remain uncertain. Nonetheless, waterborne fungi are likely associated with taste and odor problems, contamination in food and beverage preparation, and a variety of health-related effects (3, 5, 10, 17). Uncertain ramifications of fungi in potable water have led to a limited number of investigations which show that fungi are present in a significant proportion of tap water samples; however, species abundance and diversity are extremely variable (6, 11, 13, 16). This variation is often attributed to factors such as raw water source, water temperature patterns, treatment conditions, and maintenance of distribution systems (5). The nature and extent of fungi within distribution biofilms remain more obscure; only a single report has demonstrated conclusively that fungi are an integral biofilm component (12). Although it is assumed that contamination is attributable to spore deposition within the biofilm matrix, there is limited evidence to support this premise.

Pipe surfaces were collected from the Springfield, Mo., water distribution system from March to July 1997. This municipal system serves approximately 100,000 people and consists of over 900 miles of water mains. Physical and chemical characteristics of this distribution system from January to July 1997 are reported in Table 1. Each sample consisted of a single pipe coupon (i.e., a longitudinal section of the inner pipe surface) obtained during routine maintenance and extension of 30-in. mains. During collection, each coupon was carefully rinsed with 2 liters of sterile distilled water and then placed in a sterile plastic bag. Coupons were immediately transported to the laboratory and rinsed again with 1 liter of sterile distilled water. The purpose of each rinse was to minimize the likelihood of contamination by airborne spores. Although each rinse was discarded, preliminary investigations of polyvinyl chloride (PVC) and iron coupons indicated that rinses did not remove biofilm deposition.

Small biofilm fragments, each consisting of 0.5 cm^2 of surface matrix, were removed and plated on the following nonselective media: potato dextrose agar (Difco), Czapek solution agar (Difco), cornmeal agar (Difco), Sabouraud dextrose agar (Difco), and YMPG (10 g of dextrose, 5 g of neopeptone, 3 g of yeast extract, 3 g of malt extract, 10 g of agar, 1 liter distilled water). Four replicates of each medium were employed for each coupon to yield a total of 100 fragments for each coupon. The inoculated plates were placed at room temperature and monitored for growth at 24-h intervals. Fungal colonies were isolated upon formation and identified using appropriate taxonomic guides (2, 7, 20) and carbon assimilation tests (API 20 C AUX; bioMérieux Vitek, Hazelwood, Mo.).

Light microscopic observations were made for all coupons immediately following collection. Wet mounts were prepared using swabbed regions of the surface deposition that were mounted in sterile distilled water. Selected areas of four iron coupons (sites 4, 6, 7, and 8) were also examined by scanning electron microscopy (SEM). For these coupons, 1-cm^2 fragments of pipe deposition or encrustation were carefully removed from the surface matrix using a sterile dissection blade. Each fragment was chemically fixed in 2.5% glutaraldehyde in cacodylate buffer for 2 h. Specimens were then rinsed in distilled water and postfixed with 2% osmium tetroxide for 1 h. Postfixed sections were rinsed again in distilled water for 15 min and dehydrated in an ethanol series followed by further dehydration with hexamethyldisilazane for 30 min. Specimens were then air dried and mounted on an aluminum stub prior to sputter coating with gold-palladium.

Coupon characteristics and mean fungal densities for each sample are summarized in Table 2. Yeast densities were generally lower than those for filamentous fungi, as only a single coupon (site 5) exhibited a significantly greater proportion of yeast cells. Light microscopic observations of site 5, which consisted of a PVC coupon, revealed very limited surface deposition and a biofilm comprised primarily of bacteria. In contrast, all of the iron coupons exhibited extensive mineralized deposits as well as diverse microbial assemblages. Although bacteria still comprised the predominant microbial component, fungal spores and empty frustules were observed in all of the iron coupons. Examinations by light microscopy also confirmed colonization by cyanobacteria and yeasts in sites 2, 6, and 7. The presence of fungal hyphae was inconclusive on the basis of light microscopic observations; however, empty filamentous structures resembling fungal cells (3 to 10 μm) were recorded for sites 6 and 7.

Fungal isolates as well as the mean CFU per square centimeter are reported in Table 3. With the exception of cornmeal agar, all of the media utilized in this study provided suitable results. It was further determined that YMPG was the most effective medium with regard to the diversity and total number of isolates cultured. Therefore, observations from YMPG were used in reporting mean CFU per square centimeter. The onset of hyphal growth was most evident at 72 to 96 h following

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TABLE 1. Characteristics of the Springfield water distribution system (January to July 1997)

Parameter	Avg	Range
pH	7.8	7.4–8.5
Temp (°C)	16.1	6.9–28.7
Coliform bacteria (CFU)	<1	<1
Total bacteria (CFU)	1	<1–112
Free chlorine (mg/liter)	0.88	0.60–1.12
Total chlorine (mg/liter)	0.94	0.25–1.85
Turbidity (NTU[a])	0.08	<0.03–1.32
Alkalinity (mg/liter)	139	87–191
Total hardness (mg/liter)	147	98–206
Total organic carbon (mg/liter)	1.3	0.3–2.6

[a] NTU, nephelometric turbidity units.

inoculation, whereas yeast colonies were observed within 24 to 48 h. A total of 39 different species were isolated from the eight coupons. Seventeen of these isolates were cultured from two or more locations. Species of *Aspergillus* and *Penicillium* represented the most common fungi in terms of abundance (total CFU) as well as percent frequency (occurrence per site). Other frequently isolated species included *Mucor racemosis* and *Stysanus stemonites*, which occurred in three of the eight sites. Seven isolates were classified as sterile mycelium based on their lack of either sexual or asexual spores in culture. Each of these species was deemed unique based on colony and cell characteristics, and they were designated isolates A to G (Table 3). Yeast species were isolated from six samples and included *Aureobasidium pullulans*, *Candida* spp., *Cryptococcus* spp., and *Rhodotorula* spp.

Examinations by SEM indicated an extremely porous matrix comprised primarily of crystalline or scaly deposition. The ultrastructural features of the surface matrixes were very similar to those reported for other municipal distribution systems (1, 8, 14, 18). Moreover, the occurrence of bacteria within surface encrustations of Springfield's biofilms is consistent with prior findings (1, 9, 13, 19). Unique to the present study is the presence of fungal spores as a major component of surface biofilms and encrustations. Fungal spores were observed in each of the four samples and were usually loosely embedded on biofilm surfaces (i.e., biofilm-water interface) or within crevices and encrustations. Fungal spores observed by SEM were spherical and approximately 5 to 10 μm in diameter and were often associated with a unique fibrillar matrix that also harbored numerous bacteria. Although a taxonomic affiliation for these spores could not be made based on structure alone, they are of a size and shape similar to those of spores formed by *Aspergillus* and *Penicillium*, the two most common isolates in this investigation. Compared to the relatively large numbers of fungal spores, conclusive evidence for hyphal growth was not obtained by SEM analyses.

TABLE 2. Sample characteristics and mean fungal densities from pipe surfaces

Site	Service (yrs)	Surface	Deposition thickness (mm)	CFU cm^{-2}	
				Yeasts	Filamentous fungi
1	20	Iron	4	0	8.9
2	23	Iron	3	5.8	5.5
3	23	Iron	3	0	20.0
4	33	Iron	3	1.3	23.2
5	21	PVC	<1	8.9	4.0
6	70	Iron	2	7.0	24.7
7	70	Iron	2	6.6	25.2
8	73	Iron	3	5.9	14.7

TABLE 3. Fungi identified from the distribution system biofilms

Species	Site(s)	CFU cm^{-2}
Acremonium sp.	4	1.4
Alternaria alternata	7	2.2
Alternaria sp.	8	1.6
Aspergillus flavus	1, 2, 3, 6	1.9–3.6
Aspergillus sulphureus	3, 4, 6, 7	2.0–3.5
Aureobasidium pullulans	4, 8	1.3–3.1
Candida guilliermondii	7	1.7
Candida parapsilosis	5, 6	3.1–4.6
Cladosporium sp.	7	1.5
Cryptococcus laurentii	7	4.9
Cryptococcus sp.	8	2.8
Dendryphion microsporus	4	1.7
Doratomyces stemonitis	3	1.7
Gliocladium sp.	6	1.0
Mucor racemosus	3, 4, 6	2.7–3.5
Nectria sp.	8	2.8
Paecilomyces sp.	7	2.0
Papulaspora sp.	1, 4	0.84–1.1
Penicillium chrysogenum	4, 6	2.6–3.6
Penicillium citrinum	1, 3, 4, 7	1.2–3.4
Penicillium expansum	1, 4, 8	1.8–2.8
Penicillium sp.	3, 5	0.90–2.9
Penicillium sp.	3, 6, 8	0.90–3.1
Phoma sp.	7	4.3
Rhizoctonia sp.	7	2.8
Rhodotorula glutinis	2, 5	3.7–5.8
Rhodotorula mucilaginosa	6	2.4
Sporotrichum sp.	4, 8	2.0–2.8
Sporothrix sp.	1, 3	1.0–1.7
Sporothrix sp.	5	1.1
Stachybotrys chartarum	3, 6	2.8–4.8
Stysanus stemonites	2, 6, 7	2.9–4.7
Sterile mycelium A	1, 3, 4	0.50–1.6
Sterile mycelium B	2, 6	1.0–1.5
Sterile mycelium C	4	1.5
Sterile mycelium D	7	0.90
Sterile mycelium E	7	2.2
Sterile mycelium F	7	1.4
Sterile mycelium G	8	1.2

Biofilms examined in this study indicate that fungal densities and species assemblages varied considerably among the sites; however, filamentous fungi were typically more prevalent than yeasts (Table 2). Moreover, many of the genera isolated in this investigation were not observed in earlier studies that employed either tap water or biofilm samples. Conversely, several species previously isolated from treated tap water were rare or nonexistent in the Springfield distribution biofilms; however, certain soil-inhabiting genera, such as *Penicillium*, *Aspergillus*, *Mucor*, *Alternaria*, and *Cladosporium*, do appear as common colonizers of biofilms as well as potable water (11, 16). The overwhelming presence of soil-inhabiting fungi supports the paradigm that deposition is attributable to intrusion from compromised water mains or contamination during maintenance of distribution systems (5). Fungi may also survive conventional treatment strategies and enter the distribution through ineffective filter barriers (13). Indeed, observations described herein of numerous planktonic diatoms also implicate ineffective treatment and filter barriers. The influence of physical and chemical factors unique to a given distribution system should also be recognized as an important determinant of fungal deposition and growth. Water temperature would appear particu-

larly relevant in consideration of physiological constraints for spore dormancy and viability (4). Residual chlorine levels may exhibit similar constraints (15); however, information regarding the effects of chlorine on fungal spores is extremely sparse.

The results of this investigation provide further evidence that fungi are common constituents of water distribution biofilms. That fungal deposition is attributed largely to spores and not hyphal growth is significant considering that concerns such as mycotoxin production or taste and odors imply that vegetative growth occurs in situ. Nevertheless, it should be emphasized that the characteristics of a single distribution system do not discount the likelihood of vegetative growth in any given system. Differences in raw water source, treatment protocols, and system maintenance could certainly account for unique fungal assemblages. Further research should therefore elaborate on the possible mechanisms affecting spore germination and growth within distribution systems. The effects of chlorine on fungal biofilms warrant particular attention since it remains undetermined how residual chlorine levels affect spore longevity and germination.

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