Fungal biodiversity and mycotoxigenic fungi in cooling-tower water systems in Istanbul, Turkey

Duygu Göksay Kadaifciler and Rasime Demirel

ABSTRACT

This is the first study to assess fungal diversity and mycotoxigenic fungi in open recirculating cooling-tower (CT) water systems (biofilm and water phase). The production capability of mycotoxin from fungal isolates was also examined. The mean fungal count in 21 different water and biofilm samples was determined as 234 CFU/100 mL and 6 CFU/cm^2. A total of 32 species were identified by internal transcribed spacer sequencing. The most common isolated fungi belonged to the genera *Aspergillus* and *Penicillium*, of which the most prevalent fungi were *Aspergillus versicolor*, *Aspergillus niger*, and *Penicillium dipodomyicola*. From 42% of the surveyed CTs, aflatoxigenic *A. flavus* isolates were identified. The detection of opportunistic pathogens and/or allergen species suggests that open recirculating CTs are a possible source of fungal infection for both the public and for occupational workers via the inhalation of aerosols and/or skin contact.

Key words | biofilm, cooling tower, fungi, ITS-PCR, mycotoxin, thin-layer chromatography

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INTRODUCTION

Cooling-tower (CT) water systems are present in most factories, hotels, business centers, shopping centers, and residential homes. These systems are used to dispose of excess heat generated by air-conditioning systems, telecommunication devices, power plants, and other industrial systems (Figure 1). CTs are used repeatedly in water circulation systems to reduce water consumption in various fields of industry. In the process of reducing the water temperature, evaporated water is replaced with added water, usually from the municipal water supply. In open recirculating CT water systems, water is directly exposed to the atmosphere so that heat is transferred into the air. The presence of water, nutrients, and various pH and temperature zones provides a suitable environment for microbial growth. Microorganisms such as bacteria, fungi, algae, and protozoa can enter via the added water or air into the water system and form a biofilm layer on the inside surfaces of the CT (Choudhary 1998).

Nygard *et al.* (2008) reported that a CT fan can disperse bioaerosols, extending over a distance of at least 6 km from the local environment. Microfungal spores vary in diameter, mostly from 2 to 10 μm in the air, and can be carried in the air for thousands of kilometers horizontally in outdoor environments. These spores are biologically designed for easy dispersal, for example, by air movement, water movement, and insects over both short and long distances (Burge & Rogers 2000). Therefore, CTs are an ideal distribution center for fungal droplets. Inhalation of fungal spores and hyphal fragments that have allergenic and mycotoxigenic characteristics can cause serious health problems (Jarvis & Miller 2005). Besides this, fungi can be transmitted directly to the technician in contact with the water during the physical cleaning of the basin or the inside surface of the tower, which may lead to irritation of the skin (Aslund 1984).

Most previous studies have shown that hospital water systems and hemodialysis centers have been infected with

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Figure 1 | Open recirculating CTs.

Aspergillus and *Fusarium* sp. (Arvanitidou *et al.* 1999; Anaissie *et al.* 2001, 2003; Sautour *et al.* 2012). These studies concluded that water-distribution systems were a potential reservoir of opportunistic fungal infections. In addition, the existence of microfungi and their metabolites, particularly mycotoxins, could cause various negative health effects such as carcinogenic, teratogenic, and allergic reactions (Al-gabr *et al.* 2014). As a result, several studies have focused on the biodiversity of microfungi in municipal water systems in recent years (Hageskal *et al.* 2006; Pereira *et al.* 2009).

Studies concerning CTs directly connected to municipal water systems have primarily focused on the prevention of bacterial colonization or the reduction of the bacterial–protozoal load (Canals *et al.* 2015). The literature has noted that fungi can be found in cooling processes; however, there has not yet been comprehensive research on this (Morrell & Smith 1988). A few studies have focused on wood deterioration caused by micro- and macro-fungi in CTs made of wood (Morrell & Smith 1988; Schmidt *et al.* 1996). It has also been reported that fungi cause clogging in the pipelines of systems and they also have a corrosive effect on surfaces made of steel (Samimi 2013). Although fungi are significant constituents of man-made water systems, there is no recommended limit for fungal load. To the best of our knowledge, this is the first study to evaluate fungal diversity and mycotoxigenic fungi in open recirculating CT water systems.

The aims of this study were: (1) to determine fungal biodiversity in CT water systems (in the water phase and biofilm) connected to a municipal water system; (2) to identify the mycobiota by molecular and morphological methods; and (3) to determine the production capability of aflatoxin and ochratoxin by examining the secondary metabolite profiles of fungal isolates.

METHODOLOGY

Water sampling and fungal isolation

Istanbul's climate is changeable, somewhere between the Black Sea climate and the Mediterranean climate, and is therefore generally mild in character. For this reason, all kinds of plants can grow. Summer in Istanbul is hot and humid, winter is cold and rainy but rarely snowy (Turoğlu 2014). Water samples (500 mL) were collected from 21 open recirculating CTs connected to tap water in the city of Istanbul, Turkey. These 21 CTs are the only towers that we have had permission for sampling. We had only one sampling between October 2014 and June 2015. CTs' ages were reported. Water temperatures and pH values were measured. Water samples were concentrated by filtration (Sartorius, Germany) through 0.45-μm pore-sized nitrocellulose (Millipore, USA) filters. These filters were placed on Sabouraud Dextrose Agar (SDA; Oxoid, UK) plates containing the antibiotic streptomycin in duplicate and were incubated at 25 °C for 10 days (Al-gabr *et al.* 2014). After incubation, the fungal colonies were counted and colony-forming units per 100 milliliters (CFU/100 mL) were calculated. Then, the colonies were subcultured on potato dextrose agar (Oxoid) slants and stored at +4 °C.

Biofilm sampling and fungal isolation

Biofilm samples were scraped from surfaces of 10 cm^2 using a sterile scalpel and were then suspended in 10 mL sterile tap water by vortexing (Gagnon & Slawson 1999). Homogenates were serially diluted from 10^{-1} to 10^{-3}. Diluted samples of 1 mL were spreadplated and duplicated onto SDA plates with streptomycin and incubated at 25 °C for 7–10 days. After incubation, the fungal colonies were counted and CFU per square centimeter (CFU/cm^2) were

calculated. The colonies were then subcultured on potato dextrose agar (Oxoid, UK) slants and stored at +4 °C.

Morphological identification of fungi

Fungal isolates were inoculated into various media (Czapek yeast autolysate agar, Czapek-Dox agar, and Czapek yeast autolysate agar with 20% sucrose, 25% glycerol nitrate agar, malt extract agar, and potato dextrose agar) and then identified to genus and species levels according to morphological and physiological characters (Ellis 1971; Pitt 2000; Klich 2002). (All fungal author names and fungal names in this article are standardized according to the Index Fungorum website.)

Thin-layer chromatography

Fungal isolates were cultured on yeast extract sucrose agar and incubated at 25 °C for 7 days. The agar plugs with the mycelium were cut out of the colony center, the margins and edges close to other colonies, using a 6-mm diameter cork borer. The plugs were transferred to sterile screw-capped tubes and 1 mL of methanol was added. Extractions were performed ultrasonically for 15 min with sonication. A total of 20 μL of the extracts was spotted onto thin-layer chromatography (TLC) plates containing 20 × 20 cm silica gel 60 without non-fluorescence. After the spots were air dried, the TLC plates were placed in an eluent tank filled with toluene/ethyl acetate/formic acid (90%) (ratio: 5:4:1). Elution was performed for 15–30 min. After elution, the plates were air dried in a fume hood and then examined in visible light, at 366 and 312 nm, to compare ochratoxin A (OTA) and aflatoxins (AFs) (B1, B2, G1, and G2) (CAMAG HPTLC) (Frisvad & Filtenborg 1983; Samson *et al.* 2010). Fungal extracts belonging to the same species showed identical or similar secondary metabolite profiles. Therefore, the fungal isolates that showed different metabolite profiles were selected for molecular identification.

Molecular identification of fungi

Selected fungal isolates according to differences of secondary metabolite profiles were inoculated into malt extract agar and incubated at 25 °C for 7 days. Genomic DNAs were extracted from the pure cultures using a microbial DNA isolation kit (Ultraclean Microbial DNA Isolation Kit, Mobio Laboratories, Inc., USA) following the manufacturer's instructions. The standard gene regions that were the internal transcribed spacer (ITS) regions of the rDNA genes were used for molecular characterization. These regions were amplified using the primer pairs V9G, 5′-TTACGTCCCTGCCCTTTGTA-3′ (forward) and LS266, 5′-GCATTCCCAAACAACTCGACTC-3′ (reverse) (White *et al.* 1990; Samson *et al.* 2010). Polymerase chain reaction (PCR) reactions were conducted in a 25 mL final reaction volume. Each tube contained genomic 1 μL of DNA, 2.5 μL of 2.5 μM forward and reverse primers, 2.5 μL of 10× Taq buffer + KCl–$MgCl_2$ (Bioline, UK), 2.5 μL of 25 mM $MgCl_2$ (Fermentas, CA, USA), 2 μL of 2.5 mM dNTP mix, 0.25 μL of 5 U/μL Taq DNA polymerase (Bioline, UK), and 11.75 μL of sterile deionized water. DNA amplification was performed in a thermocycler with an initial denaturation step for 5 min at 95 °C, followed by 35 cycles of denaturation for 45 s at 95 °C, annealing for 30 s at 56 °C with an extension of 2 min at 72 °C. A final extension at 72 °C was performed for 6 min (White *et al.* 1990; Samson *et al.* 2010). To confirm the amplification of solely the ITS, 5 μL of PCR product together with the marker (GeneRuler DNA Ladder 50 bp, Fermentas) was resolved by gel electrophoresis on 1% agarose gel containing 5 μg/mL GelRed in 1× TAE buffer. The gel samples were photographed via the Gel Documentation System (Uvitec M02 4611). The PCR products were cleaned up using EXOSAP-IT (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and used for sequencing.

The ITS regions were sequenced using ITS1-TCCGTAGGTGAACCTGCGG (forward) and ITS4-TCCTCCGCTTATTGATATGC (reverse); sequencing reactions were performed with the CEQ™ DTCS Quick Start Kit (Beckman Coulter, CA, USA) and sequenced by the CEQ™ 8000 Genetic Analysis System. The sequences were allocated GenBank accession numbers and compared with those deposited in the NCBI GenBank database.

Statistical analyses

Statistical analyses were carried out using a Spearman's correlation coefficient test (IBM SPSS, version 21, USA). The

test was used to examine the relationship of fungal counts (in water and biofilm) with selected parameters such as temperature, pH and CTs's age. Significant differences were considered at $p < 0.05$.

RESULTS

Distribution of fungi in water and biofilm samples

Fungal counts in the 21 CT water samples counted ranged from 21 to 1,070 CFU/100 mL. The highest fungal count was detected in CT 4. Fungi could not be detected in one of the CT water samples (CT 21). In the biofilm samples, fungal counts ranged from 1 to 12 CFU/cm^2. The highest and the lowest fungal counts of biofilm samples were detected in CT 10 and CT 15, respectively (Table 1).

The values of the water temperature ranged from 20 to 33 °C (mean 28 °C) and the pH values ranged from 7 to 9 (mean 8). The ages of CTs ranged from 1 month to 16 years (Table 1). The Spearman correlation coefficient test indicated no significant relationship between fungal counts (in water and biofilm) and the above parameters (temperature and pH), respectively ($p = 0.116$, $r = -0.353$ in water/temperature; $p = 0.788$, $r = -0.063$ in biofilm/temperature; $p = 0.074$, $r = -0.398$ in water/pH; $p = 0.244$, $r = -0.266$ in biofilm/pH). A positive correlation between fungal concentrations (in water and biofilm) and CTs' age ($p = 0.023$, $r = 0.495$ for water; $p = 0.027$, $r = 0.481$ for biofilm) was determined.

Thin-layer chromatography

Fungal extracts belonging to the same species showed identical or similar secondary metabolite profiles. The results revealed that 68 out of 224 isolates showed different metabolite profiles, and these isolates were selected for molecular identification. On the other hand, when the metabolite profiles of all isolates were compared for production capability of OTA and AFs (B1, B2 and G1, G2) with mycotoxin standards, 14 isolates (5%) were determined to be capable of aflatoxin producing AFs (B1, B2). These 14 aflatoxigenic *Aspergillus flavus* were isolated from nine different towers (CT 3, 8, 9, 13, 16, 17, 18, 19, and 20). While 11 of 14 aflatoxigenic *A. flavus* isolates were identified from water samples, three of them were isolated from biofilms. Aflatoxigenic *A. flavus* isolates were identified from both biofilm and the water samples of CT 16 and 17 (Table 2).

Identification of fungal isolates

A total of 224 fungal isolates were subcultured, 141 and 83 fungal colonies from water and biofilm samples. Of these, 68 isolates were selected for molecular identification, carried out using DNA sequencing (ITS region). Comparing the results of morphology and molecular identification, a total 32 species were identified belonging to 15 genera by ITS-PCR, while a total of 18 species were identified belonging to 9 genera by classical culture methods (Table 2).

Fungal diversity in molecular identification was higher than in morphological identification. When the morphological identification results are examined in Table 2, it can be seen that 18 (56%) out of 32 species and 9 (60%) out of 15 genera had identical molecular identification results. In addition, six fungi (40%) were not identified by classical culture methods, rather only identified by PCR-based methods. The most common isolated fungi belonged to the genera *Aspergillus* and *Penicillium*, of which the most prevalent fungi in the water samples were *A. versicolor* with 420 CFU/100 mL and *A. niger* with 219 CFU/100 mL. The most prevalent fungi in biofilm samples were *A. sydowii* with 17 CFU/cm^2. While 29 fungal species were isolated from water samples, 24 fungal species were isolated from biofilm samples. Unlike the biofilm fungi in water samples, *Fusarium* sp., *Penicillium simplicissimum*, *P. oxalicum*, *Trichoderma longibrachiatum*, *Mucor circinelloides*, *Purpureocillium lilacinum*, *Talaromyces radicus*, *A. amstelodami*, *A. flavipes*, and *P. carneum* were determined. *P. gladioli*, *A. terreus*, *Coniochaeta ligniaria*, *A. tamarii*, and *Coprinellus* sp. were determined in the biofilm samples. *Coprinellus* sp. spores (belonging to Basidiomycetes) were isolated from only one tower. Among the fungal isolates, *A. fumigatus* (37) and *A. flavus* (26) had the highest number of isolates. Aflatoxigenic *A. flavus* was present in both water and biofilm samples of CT 16–20. *A. fumigatus* was present in both water and biofilm samples of CT 8, 10–13, 15, 16, and 20.

Table 1 | The results of fungal concentrations (water and biofilm) and parameters in 21 CT water systems

CT code (No.)	CT age (year)	Sampling time	Temperature (°C)	pH	Water (CFU/100 mL)	Biofilm (CFU/cm^2)
CT 1	16	October 2014	20	8	265	5
CT 2	16	October 2014	21	7	900	8
CT 3	15	October 2014	23	8	76	4
CT 4	16	October 2014	25	7	1070	7
CT 5	16	October 2014	25	7	700	2
CT 6	15	April 2015	29	9	21	10
CT 7	16	April 2015	28	9	437	4
CT 8	16	April 2015	27	8	116	1
CT 9	15	April 2015	24	8	69	1
CT 10	15	April 2015	26	7	111	12
CT 11	3	May 2015	30	9	139	1
CT 12	8	May 2015	29	8	210	3
CT 13	10	May 2015	29	8	227	5
CT 14	10	May 2015	32	7	230	6
CT 15	1 month	May 2015	28	8	60	1
CT 16	15	June 2015	31	8	73	2
CT 17	15	June 2015	29	8	82	3
CT 18	10	June 2015	31	7	31	1
CT 19	16	June 2015	33	8	53	45
CT 20	10	June 2015	33	8	40	1
CT 21	1 month	February 2015	24	9	0	1
Mean			28	8	233	6

CT, cooling tower; CFU/100 mL, colony-forming units per 100 mL; CFU/cm^2, colony-forming units per square centimetre; °C, Celsius.

DISCUSSION

Previous investigations have indicated that water-distribution systems can disseminate potentially allergenic, mycotoxigenic, and opportunistic fungi in medical and non-medical buildings (Sautour *et al.* 2012). Direct contact of water with skin or wounds and inhalation of bioaerosols originating from water-distribution systems can lead to keratitis, skin allergies, asthma and various respiratory problems, or fungal infections. Therefore, regular microbiological monitoring of man-made water systems is important for public health. However, limited attention has been given to the presence of fungi in CT water systems (Morrell & Smith 1988; Schmidt *et al.* 1996). Qualitative and quantitative assessments of fungi in CTs relating to health significance have yet to be conducted. To our knowledge, this study is the first to report on problems with fungal biodiversity and mycotoxigenic fungi in different water and biofilm samples of CT systems in Turkey. In the present study, fungal counts in water samples of 21 CTs ranged from 0 to 1,070 CFU/100 mL. The mean fungal count was determined as 233 CFU/100 mL. While the highest fungal count was detected in CT4 in October, the lowest fungal counts were in CT21 in February (Table 1).

Meteorological parameters such as wind, rainfall, and also vegetation are associated with fungal growth and dispersal in air. In winter, the temperature is too low for fungal development and because there is less food available. Therefore, airborne fungi are more abundant in autumn than winter in Turkey because of the favorable conditions (Aydogdu *et al.* 2010). Seasonal aeromycoflora levels could affect the CT contamination levels because CT is directly exposed to the atmosphere so that airborne fungi may contaminate the water. Therefore, we suggested that

Table 2 | The number of identified fungi isolated from water and biofilm samples in different CTs

Fungal biodiversity (morphological identification)	Fungal biodiversity (molecular identification)	Closest blast hit (identity %/coverage %)	GenBank accession number	Fungarium codes of isolates	CT code (No.)	Total fungal concentration (CFU/100 mL)	Total fungal concentration (CFU/cm^2)
Alternaria alternata (Fr.) Keissl. 1912	*Alternaria alternata* **(Fr.) Keissl. 1912**	99/87	KX090314	**B74**, **W138**, W71, W113, W124	CT 7, 11, 12, 13	124	1
		97/98	KX090333				
Aspergillus amstelodami Thom & Church 1926	*Aspergillus amstelodami* **Thom & Church 1926**	96/96	KX090342	**W23**, **W76**, **W410**, W23A,	CT 2, 4, 7	210	0
		98/87	KX090349				
		96/97	KX090347				
Aspergillus sp. P. Micheli ex Haller 1768	*Aspergillus flavipes* **(Bainier & R. Sartory) Thom & Church 1926**	87/90	KX090343	**W22**	CT 2	150	0
**Aspergillus flavus* Link 1809	**Aspergillus flavus* **Link 1809**	96/99	KX090296	**W193**, **W195**, **W1310**, W89, W182, W191, B185, B187, B191, B193, B205, B206, B161, B171, B175, W31, W88, W910, W162, W163, W164, W174, W181, W1311, W201, W67	CT 3, 6, 8, 9, 13, 16, 17, 18, 19, 20	79	4
		99/98	KX090295				
		99/99	KX090332				
Aspergillus fumigatus Fresen. 1863	*Aspergillus fumigatus* **Fresen. 1863**	99/97	KX090299	**W2010, B83, B103, W114, W1011, W155A, W151, W48, W814, B173, B81,** B72, B124, B125, W144, W169, W62, W123, W139, W141, B77, B82, B113, B122, B134, B178, W108, B71, B151, W156, B207, W121, W135, B153, B73, W154, B194	CT 4, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20	160	5
		99/96	KX090311				
		98/98	KX090319				
		99/74	KX090325				
		98/98	KX090326				
		98/98	KX090334				
		99/100	KX090336				
		99/99	KX090348				
		94/96	KX090350				

		99/98	KX090352				
		98/98	KX090357				
Aspergillus niger Tiegh. 1867	*Aspergillus niger* **Tiegh. 1867**	87/91	KX090292	**W197**, W24, B41, W79, W131, W161, B172, B186, W204	CT 2, 4, 7, 13, 16, 17, 18, 19, 20	219	7
Aspergillus sydowii (Bainier & Sartory) Thom & Church 1926	*Aspergillus sydowii* **(Bainier & Sartory) Thom & Church 1926**	99/95	KX090304	**B65**, W61, B62, B65A, B112, B123, B133, B135, B141	CT 6, 11, 12, 13, 14	8	17
Aspergillus sp. P. Micheli ex Haller 1768	*Aspergillus tamarii* **Kita 1913**	99/93	KX090354	**B204**, B198, B203	CT 19, 20	0	1
Aspergillus terreus Thom 1918	*Aspergillus terreus* **Thom 1918**	99/93	KX090315	**B101**, B91	CT 9, 10	0	1
Aspergillus versicolor (Vuill.) Tirab. 1908	*Aspergillus versicolor* **(Vuill.) Tirab. 1908**	99/96	KX090291	**W198, W213, W211, B76, W29, W16, W55,** W1210, W184, W171, B25, B162, B199	CT 1, 2, 3, 5, 7, 11, 12, 16, 18, 17, 19	420	1
		99/98	KX090300				
		98/97	KX090301				
		99/96	KX090312				
		98/99	KX090341				
		98/97	KX090344				
		99/99	KX090346				
Chaetomium sp. Kunze 1817	*Chaetomium iranianum* **Asgari & Zare 2011**	99/86	KX090316	**B111,** W111, W116, W117, W1110, W136, W153, B85	CT 8, 11, 13, 15	182	1
Non identified	*Circinella muscae* **(Sorokīn) Berl. & De Toni 1888**	97/98	KX090298	**W175,** W149, W214, B131, B1910, B202, W215, W911	CT 2, 9, 13, 14, 17, 19, 20	61	1
Cladosporium cladosporioides (Fresen.) G.A. de Vries 1952	*Cladosporium cladosporioides* **(Fresen.) G.A. de Vries 1952**	90/94	KX090309	**B22,** B68, W128, B142, W126, B107, W148, B182, B152, W93, B181	CT 2, 6, 9, 10, 12, 14, 15, 18	136	3
Non identified	*Coniochaeta ligniaria* **(Grev.) Cooke 1887**	93/99	KX090317	**B121**	CT 12	0	1
Non identified	*Coprinellus sp.* **P. Karst. 1879**	92/96	KX090356	**B209**	CT 20	0	1

(*continued*)

Table 2 | continued

Fungal biodiversity (morphological identification)	Fungal biodiversity (molecular identification)	Closest blast hit (identity %/coverage %)	GenBank accession number	Fungarium codes of isolates	CT code (No.)	Total fungal concentration (CFU/100 mL)	Total fungal concentration (CFU/cm^2)
Fusarium sp. 1 and 2. Link 1809	*Fusarium sp.*1 and 2. **Link 1809**	91/96	KX090294	**W185, W1610,** W81, W86	CT 8, 16, 18, 19, 20	40	0
		92/96	KX090339				
Fusarium oxysporum Schltdl. 1824	*Fusarium oxysporum* **Schltdl. 1824**	97/90	KX090307	**B33, S145,** W36, W133, W75, B31, B32, B136, B176, W1293, W146A, W146, B192	CT 3, 7, 12, 13, 14, 17, 19	181	8
		97/97	KX090331				
Fusarium sp. Link 1809	*Fusarium solani* (**Mart.**) **Sacc. 1881**	97/99	KX090303	**W102, W203,** B195, W199, W164A, W165, B164, W173, B177	CT 10, 16, 17, 19, 20	60	1
		97/99	KX090355				
Non identified	*Mucor circinelloides* **Tiegh. 1875**	95/74	KX090328	**W122**, W68, W69, W99	CT 6, 9, 12	14	0
Paecilomyces variotii Bainier 1907	*Paecilomyces variotii* **Bainier 1907**	98/99	KX090297	**W183, W64,** B66, W205, B201, B1710	CT 6, 17, 18, 20	10	1
		94/100	KX090345				
Non identified	*Paraconiothyrium fuckelii* (**Sacc.**) **Verkley & Gruyter 2012**	99/99	KX090310	**B84,** W33,W77, W84, W94, B105, W118, B179	CT 3, 7, 8, 9, 10, 11, 17	77	9
Penicillium sp. Link 1809	*Penicillium carneum* (**Frisvad**) **Frisvad 1996**	98/99	KX090358	**W103**	CT 10	10	0
Penicillium chrysogenum Thom 1910	*Penicillium chrysogenum* **Thom 1910**	99/97	KX090329	**W1112, W155, W172,** W25, B92,	CT 2, 9, 11, 15, 17	190	1
		98/98	KX090335				
		97/96	KX090338				
Penicillium citrinum Thom 1910	*Penicillium citrinum* **Thom 1910**	96/98	KX090322	**B106, W115,** W52, W63, W710, W711, W85, W811, W97, B165	CT 5, 6, 7, 8, 9, 10, 11, 16	130	1
		99/97	KX090324				
Penicillium commune Thom 1910	*Penicillium commune* T**hom 1910**	99/98	KX090321	**W104,** W17, W812, W813, W105, W119, B184	CT 1, 8, 10, 11, 18	56	1

Penicillium sp. Link 1809	*Penicillium dipodomyicola* **(Frisvad, Filt. & Wicklow) Frisvad 2000**	99/98	KX090305	**B64, B61, B102, B208,** W78, W712, W26, W82, W91, W167, B51, B52, W72, W95, B174, W127	CT 2, 5, 6, 7, 8, 9, 10, 12, 16, 17, 20	201	5
		99/98	KX090306				
		99/99	KX090318				
		98/98	KX090353				
Penicillium sp. Link 1809	*Penicillium gladioli* **Machacek 1927**	93/98	KX090308	**B23**	CT 2	0	3
Penicillium oxalicum Currie & Thom 1915	*Penicillium oxalicum* **Currie & Thom 1915**	99/98	KX090323	**W1111**, W44, W45	CT 4, 11	30	0
Penicillium simplicissimum (Oudem.) Thom 1930	*Penicillium simplicissimum* **(Oudem.) Thom 1930**	94/97	KX090302	**W210, W147**	CT 2, 14	30	0
		95/99	KX090337				
Penicillium spinulosum Thom 1910	*Penicillium spinulosum* **Thom 1910**	99/99	KX090293	**W196, B75,** B197	CT 7, 19	10	1
		99/88	KX090313				
Non identified	*Purpureocillium lilacinum* **(Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson 2011**	99/99	KX090330	**W18**, W145A	CT 1, 14	1	0
Talaromyces sp. C.R. Benj. 1955	*Talaromyces radicus* **(A.D. Hocking & Whitelaw) Samson, N. Yilmaz, Frisvad & Seifert 2011**	95/99	KX090340	**W35**	CT 3	10	0
Trichoderma sp. Pers. 1794	*Trichoderma harzianum* **Rifai 1969**	97/99	KX090320	**B166, W109,** W107, B168	CT 10, 16	13	1
		97/92	KX090351				
Trichoderma longibrachiatum Rifai 1969	*Trichoderma longibrachiatum* **Rifai 1969**	99/99	KX090327	**W132**, W98, W134	CT 9, 13	6	0

CT, cooling tower; CFU/100 mL; colony-forming units per 100 mL; CFU/cm^2, colony-forming units per square centimetre, sequenced isolated shown in bold font.
*.aflatoxigenic fungi.

to determine the CT contamination levels, sampling should be performed at monthly intervals during the year.

The CTs' age can also be associated with fungal counts. This explains why older towers (i.e., CT 4) have higher fungal counts than new towers (i.e., CT 21) according to correlation results. Anaissie *et al.* (2003) discussed how mature biofilm in older buildings breaks into fragments that can be released into the water flow. Microorganisms thus spread throughout water systems, increasing the level of microbial counts. Several studies have surveyed the quantification of fungi in municipal water from public networks, hospitals, houses, and tanks, reporting that fungal counts ranged from 1 to 300 CFU/100 mL (Arvanitidou *et al.* 1999; Hageskal *et al.* 2006; Grabinska-Łoniewska *et al.* 2007; Al-gabr *et al.* 2014). These differences may be explained by different approaches: (1) different usage of disinfection procedures at the municipal water system, (2) the age of the water-distribution systems, and (3) the influence of temperature and pH conditions. All the above factors are important for fungal deposition and growth. Although municipal water is chlorinated, it is considered that the level of free chlorine decreases in the water as the water flows through miles of pipelines. The chlorination is thus not sufficient for the eradication of microorganisms. Moreover, the concentration of chlorine used for disinfection and the variation in the disinfection periods may cause serious modifications in the type and number of microorganisms. Siqueira & Lima (2011) reported that while free fungal spores were susceptible to high concentrations of free chlorine, fungal biofilms were resistant. However, additional studies have reported that fungal spores are much more resistant to chlorine than are biofilm or fungal mycelia (Doggett 2000; Grabinska-Łoniewska *et al.* 2007; Sautour *et al.* 2012). Different disinfectants were used in addition to chlorine in the towers in this present study; however, technicians were unable to provide information on which biocides were preferred or how they were used. Our findings, consequently, may not show exactly the total number of fungi. Either most of the microorganisms were eradicated by biocides or they may have acquired resistance to biocides. Nor can we say whether chlorination alone is or is not sufficient. Furthermore, the conventional culture methods can be implemented only by the number of colonies of living cells. When the microorganisms are exposed to chemical agents such as disinfectants, some of them lose their viability and some of them can form viable but non-culturable cells. Viable but non-culturable cells that can proliferate under suitable conditions can be detected by independent culture methods such as tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining. Therefore, the total number of living cells can actually be greater than the number obtained (CFU/100 mL) by culture methods (Chi & Li 2007). In future study, it is planned to use CTC staining technique to evaluate the disinfectant effectiveness of microorganisms in water systems. Anaissie *et al.* (2003) considered the age of the water-distribution system to be important for microbial load. It was suggested that the plumbing systems of older buildings further supported biofilm formation. Although the primary source of fungi is soil, fungi are also well adapted to water systems. Physical conditions are essential for fungal growth and mycotoxin production. Although there is a great variation in the response of fungi to temperature, they often prefer mesophilic temperatures. It is best to select a temperature between 20 and 30 °C. Most fungi grow and produce mycotoxin optimally in an acidic environment with pH between 5 and 6; however, they generally show good growth in a wide pH range from 3 to 8 (Barnett & Hunter 1999; Doggett 2000). In this study, the mean values of temperature and pH were detected as 28 °C and pH 8.

There are a limited number of studies concerning fungal biofilm in municipal water systems (Doggett 2000; Anaissie *et al.* 2003; Grabinska-Łoniewska *et al.* 2007). Indeed, this study is the first to collect biofilm samples from different CT water systems connected to municipal water. In the present study, fungal counts in 21 CT biofilm samples ranged from 1 to 12 CFU/cm^2.

While Doggett (2000) reported that fungal counts ranged from 4 to 25 CFU/cm^2, other studies have only examined the existence and biodiversity of fungi in biofilm. Water-distribution systems are generally made of iron, polyvinyl chloride, copper, or steel. Previous studies have indicated that physical and chemical interactions with the pipeline surfaces affect biofilm attachment. Doggett (2000) and Grabinska-Łoniewska *et al.* (2007), respectively, demonstrated that iron surfaces show a higher density of fungi than polyvinyl chloride and steel surfaces. In the present study, all the CTs were made of steel. Therefore, depending on the

materials, various effects on biofilm formation and microbial growth can be observed. In addition, the sampling area was protected from disinfectant residuals (Prest *et al.* 2016).

The age of the sampling area is a significant factor for fungal growth. In the present study, it was indicated that high fungal levels in the biofilm samples were detected in the older CTs (i.e., CT 10) compared to new CTs (i.e., CT 15).

Different usage of disinfectants (especially, that is, dosage and type) could affect the numbers of fungi in both the water and the biofilm in different systems (i.e., CTs and municipal distribution systems). It is recommended that disinfectants do not adversely affect human health and do not cause corrosion on surfaces (Lin *et al.* 1996). For this reason, in a subsequent study, it is planned to assess the efficacy of copper-silver ionization as an alternative to chlorine in controlling the biofilm formation and planktonic microorganisms at constant conditions in a simulated recirculating CT system.

Previous studies have shown greater fungal diversity in both biofilm and water-distribution systems. The most common isolated fungi from biofilm and water-distribution systems were identified as the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Exophiala*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus*, and *Trichoderma*, most of which can produce large amounts of spores that are easily dispersed and have the ability to travel long distances. These spores can penetrate the nose and the upper or lower respiratory tract, exposing people to various health problems, such as allergies, asthma, and mycoses. The importance of mycotoxigenic fungi in water-distribution systems is not completely understood. The present study is the first to conduct a mycotoxin search in CT water systems. Even though mycotoxins in water may be considered extremely diluted, it has been suggested that small amounts of mycotoxin exposure in the long term could lead to serious problems in the human body (Al-gabr *et al.* 2014). Although direct skin contact or inhalation of toxins is believed to be of minor importance for the general population, still attention has been drawn to these transmission methods (Jarvis & Miller 2005; Boonen *et al.* 2012). The general characteristics of the medically important species identified in this study (Table 2) are described as follows. *A. flavus*, *A. flavipes*, *A. fumigatus*, *A. niger*, *A. tamarii*, *A. terreus*, and *A. sydowii* are frequent causative agents of allergic pulmonary disease, mycotic keratitis, and invasive and non-invasive aspergillosis. Moreover, *A. flavus*, *A. niger*, *A. terreus*, and *A. versicolor* are known to be important mycotoxin producers (Jarvis & Miller 2005; Samson *et al.* 2010). In this study, 14 aflatoxigenic *A. flavus* isolates were detected in 42% of the surveyed CTs. It has been reported that *Aspergillus* spp., which have allergic and/or toxigenic properties, such as *A. flavus*, *A. fumigatus*, *A. versicolor*, and *A. niger*, are prevalent inhabitant fungi. Therefore, it is considered that open recirculating CTs could be possible sources of opportunistic fungal diseases for both the public and occupational workers via inhalation of aerosols and/or skin contact. While the prevalent fungi in water samples were *A. versicolor* and *A. niger*, *A. sydowii* was predominant in biofilm samples. Most of the fungi were identified as being present in both biofilm and the water phase (Table 2). It has been considered that mainly water-phase microorganisms are a primary source of biofilm. Each phase forms its own microbial community with different species under specific environmental parameters. The detachment of biofilm fragments could contribute to the variation in the quantity and quality of microbiota. Studies on the interactions between microorganisms in water and biofilm are still being performed (Prest *et al.* 2016). Based on the morphology and molecular identification results, it is suggested that molecular identification confirms the classical morphological identification and also demonstrates the diversity of species. The ITS primers are regarded as universal primers for fungi. Indeed, Al-gabr *et al.* (2014) reported that ITS primers were suitable for assessing fungal diversity in water. In addition, non-culturable fungi are important to understand the mycoflora in aquatic environments and the ecology of biofilms. Denaturing gradient gel electrophoresis could provide an idea of community profiles (Pereira *et al.* 2010) and hence it is planned to be used as a complementary technique of culture-based methods.

CONCLUSIONS

This is the first study in which mycobiota in open recirculating CT systems (biofilm and water phase) and the production capability of aflatoxin and ochratoxin of fungal

isolates have been researched. The remarkable findings are listed below:

- The counts of fungi recorded in the water samples ranged from 21 to 1,070 CFU/100 mL and from 1 to 12 CFU/cm^2 in the biofilm samples.
- From the 224 fungal strains isolated, 32 species were identified, belonging to 15 genera, as identified by ITS sequencing.
- The most common isolated fungi belonged to the genera *Aspergillus* and *Penicillium*, of which the most prevalent were *A. versicolor*, *A. niger*, and *P. dipodomyicola*.
- Fourteen aflatoxigenic *A. flavus* strains were isolated from 42% of surveyed CTs.
- The detection of *Aspergillus*, *Penicillium*, and *Fusarium* species suggests that the open recirculating CTs are a possible source of infection.

Culture-independent techniques as a complementary technique should be used to determine both the fungal community and total fungal concentration in environmental samples.

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