FUNGAL ECOLOGY AND DISINFECTION IN DRINKING WATER SYSTEMS

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Drinking water is far from a sterile environment and hosts a complex microbial community, including bacteria, eukaryotes, and viruses. Previous research regarding drinking water microbiology has been focused on bacteria. Fungi are commonly found microorganisms in drinking water; however, fungal ecology in drinking water is poorly understood, because of both limited amount of studies and methodological limitations of culture methods. Centralized water treatment processes, which may include coagulation-sedimentation, media filtration, and disinfection, has been suggested as a threshold shaping bacterial community structure in treated drinking water. In addition, a holistic knowledge of microbial ecology within the centralized drinking water treatment process could enable updated water microbiological quality control. On-site addition of secondary disinfectant to premise plumbing has been used to eliminate opportunistic pathogens, primarily *Legionella*. However, the effect of disinfection on fungal ecology has not been evaluated in detail, hindering the holistic understanding of drinking water microbial ecology.

This dissertation is focused on fungal ecology in relation with centralized drinking water treatment process and premise plumbing secondary disinfection; free chlorine and monochloramine disinfection kinetics of waterborne fungi were also evaluated. In the current study, fungal abundance and community structure change along drinking water treatment processes were evaluated using culture-independent methods including qPCR and next

generation sequencing (NGS). Fungal abundance and community structure was found to be significantly affected by the media filtration step, but the disinfection step did not demonstrate significant effects on fungal ecology. NGS was also applied to evaluate fungal community structure change in a hospital premise plumbing system treated with on-site monochloramine disinfection. No significant change in the fungal community structure was observed before and after the initiation of on-site disinfection, although the on-site disinfection was previously found to shift bacterial community structure. Fungal community was found to be dominated by phyla Ascomycota, with *Penicillium* and *Aspergillus* were the most frequently detected genera.

Free chlorine and monochloramine disinfection kinetics of *Penicillium* and *Aspergillus* were evaluated. The tested *Penicillium* and *Aspergillus* strains were found to be disinfection resistant. The observed inactivation data were fitted to the delayed Chick-Watson model, and the required Ct values (product of disinfectant concentration C $\times$ contact time t) for 3-$\log_{10}$ inactivation of the tested fungi ranged 48.99 mg•min/L to 194.7 mg•min/L for free chlorine; and ranged from 90.33 mg•min/L to 531.3 mg•min/L for monochloramine. The 3-$\log_{10}$ inactivation Ct values for fungi are within the similar range for *Legionella* and *Mycobacterium*, which are common waterborne, disinfection resistant bacteria. The resistance to disinfection by *Penicillium* and *Aspergillus* could facilitate the survival in treated municipal drinking water.

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1.0 DISSERTATION INTRODUCTION

1.1 MOTIVATION AND OBJECTIVES

1.1.1 Drinking water as an ecological niche for fungi

The drinking water system, from treatment plant to premise plumbing, is highly complex and hosts a diverse microbial community. Fungi are common waterborne microorganisms, but little is known about the drinking water fungal diversity and community dynamics in drinking water systems. Previous culture-dependent research demonstrated wide occurrence of fungi in drinking water [1-3]; however, the fungal ecology in drinking water is still poorly understood, due to both a limited amount of research and previous methodological limitations [4].

Diverse fungal species inhabit natural surface and ground water [5, 6], previous research has also shown that fungi can pass through sand filtration and free chlorine disinfection [7], and diverse fungal species have also been previously isolated from treated drinking water and water pipe biofilm samples [2, 3, 8, 9]. Previous studies of drinking water fungi have been primarily culture-based, limiting the number of fungal isolates that may be identified [4]. The culture-based identification of fungi depends on morphological identification, which relies on researchers' knowledge and experience, and the fact that many fungi that cannot grow or sporulate under laboratory conditions make morphological identification impossible [4].

Isolation procedures also vary from study to study, causing differential detection sensitivity [4, 5]. The variation in medium and culture temperature has also been found to cause difference in detection sensitivity and selectivity towards certain species [4, 5]. These challenges have limited the full evaluation of the fungal ecology in drinking water.

Next generation sequencing (NGS) has vastly expanded researchers' ability to investigate complex microbial ecology in the environment [10, 11], most commonly using amplicon sequencing of the 16S rRNA gene region to investigate bacterial communities [12-15]. Due to its culture-independent nature, NGS enables researchers to overcome limitations posed by culture methods such as unintended selection by culture media [4, 16]. Although inherently limited by being not able to differentiate viable and non-viable microorganisms [15, 17], NGS enables high-throughput and high-resolution characterization of the microbial community including viable but non-culturable microorganisms, which are the majority of environmental microbial community, making it suitable for evaluating complex microbial community structure dynamics in environment such as drinking water [15, 16, 18]. Amplicon sequencing of the 18S rRNA gene region has been previously used to investigate the ecology of drinking water eukaryotes [19, 20]. The internal transcribed spacer (ITS) has been suggested as the primary gene marker for fungi, enabling more detailed fungal ecology study [21, 22].

Opportunistic pathogens in drinking water have emerged as a public health issue. Although generally not a risk for healthy individuals, opportunistic pathogens may infect immunocompromised persons and result in significant morbidity and mortality, currently posing the greatest infectious risk from drinking water in the industrialized countries [18]. As total elimination of microorganisms from drinking water is unrealistic using current technologies, a holistic understanding of drinking water microbial ecology is imperative for updating the current

waterborne infectious risk control [12, 16, 18, 23]. Pathogenic fungi, such as *Aspergillus* spp. and *Fusarium* spp., have also been found in drinking water systems [24, 25]; however, drinking water as a potential route of transmission for pathogenic fungi has not yet been widely recognized and evaluated. Currently there is no regulation regarding drinking water fungi in the United States. Researchers have suggested that nosocomial fungal infection can be waterborne and precautionary measures should be taken [24, 26], while others argued that further studies are needed [4, 27, 28]. The ecology of drinking water fungi is poorly understood, and a major source of controversy in interpreting the role of drinking water fungi in opportunistic fungal infections [4].

1.1.2 Drinking water treatment shapes the microbial community

Generally, drinking water is first treated by a centralized water treatment process, which includes coagulation-sedimentation, media filtration, and disinfection, before distributing to end-point users [16]. Research has shown that centralized drinking water treatment processes, particularly the media filtration step, acts as a threshold shaping bacterial community structure in treated drinking water [15, 29]. Disinfection was also shown as a factor shaping bacterial community [14, 30] (Figure 1.1). It has been suggested that the treatment process controls microbial ecology in drinking water, and knowledge of each treatment step's effect on microbial community structure could enable updated water microbiological quality control, in ways such as managing microbial community structure to minimize opportunistic pathogen risk [15, 16].

Addition of secondary disinfection to premise plumbing has been adopted as an approach to minimize opportunistic pathogen infections such as *Legionella* spp. [31, 32] (Figure 1.1).

Currently, the effect of disinfection on waterborne microbial ecology has only been evaluated to a limited degree [12, 31]. Studies found that secondary disinfection shifted the bacterial community structure, enriching the relative abundance of bacterial groups such as Firmicutes, Alphaproteobacteria, and Gammaproteobacteria [12, 31].

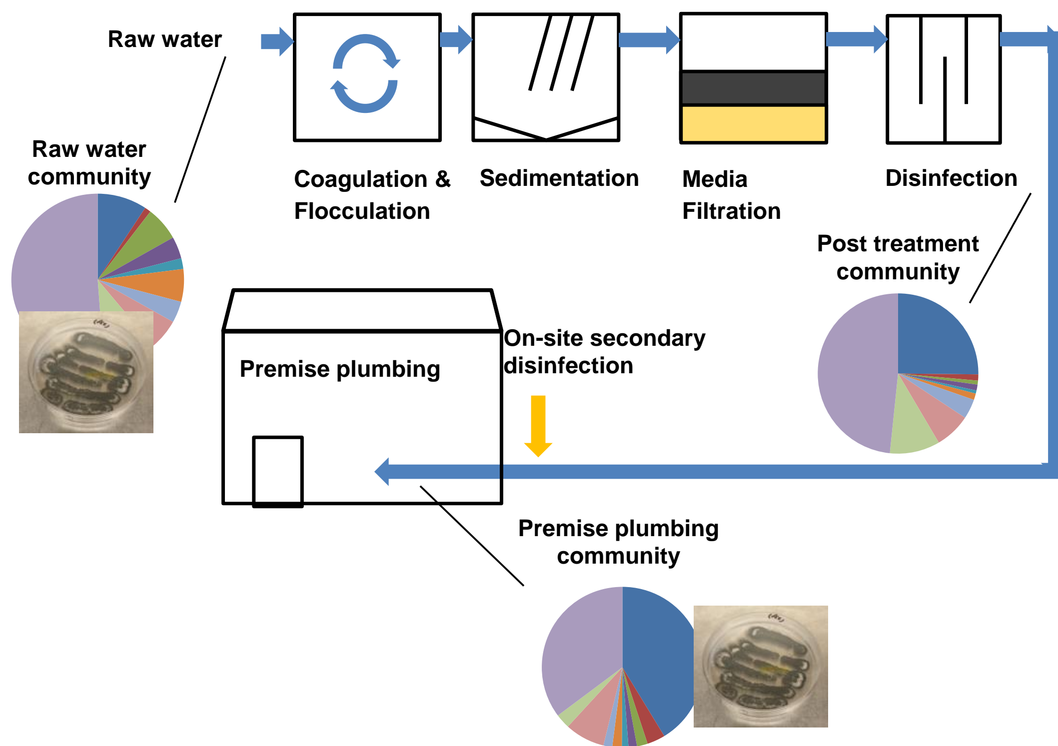

Figure 1.1 Conceptual overview of microbial community structure change from raw water to premise plumbing system

Despite the fact that fungi are widely occurring microorganisms in drinking water and potentially contain notable opportunistic pathogens, fungal diversity and community structure dynamics in drinking water are poorly understood. The diversity of fungi in drinking water has been evaluated by a limited amount of research, and fungal community dynamics during centralized drinking water treatment and in relation to secondary disinfection in premise plumbing system are still unknown. Disinfection kinetics are essential to estimate microbial survival in treated drinking water and associated risks [33]. The resistance of waterborne fungi to disinfection has been investigated by a limited number of studies [34-36], but detailed evaluation of disinfection kinetics of fungi is still lacking.

The current dissertation research is aimed to investigate fungal diversity and community structure associated with centralized drinking water treatment and premise plumbing secondary disinfection, as well as the disinfection kinetics of fungi to expand the body of knowledge relevant to drinking water microbiology.

1.2 DISSERTATION ORGANIZATION

The objectives of this presented research are accomplished through four research projects, which are incorporated in four manuscripts for journal publication. The dissertation research is presented in the following chapters.

Chapter 2: Literature Review of Fungal Diversity in Drinking Water Systems

Chapter 2 presents a literature review of fungal ecology in drinking water systems. The scope of the literature review was commonly isolated fungal species, emerging culture-independent techniques for studying fungal ecology in drinking water, disinfection of fungi, and potential human health significance of drinking water fungi. By comparing different studies evaluating presence of fungi in drinking water, this chapter summarized that *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma* were the most commonly isolated fungal genera. The study also found the spectrum of commonly isolated fungi contains several opportunistic fungal pathogens, including *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus flavus*, *Fusarium solani*, and *Fusarium oxysporum*. Current knowledge gaps regarding lack of evaluation of fungal ecology and disinfection kinetics in drinking water were also identified.

Chapter 3: Drinking Water Fungal Community Structure Shaped By Centralized Drinking Water Treatment Unit Operations

Chapter 3 evaluates the fungal community structure along a conventional centralized drinking water treatment process, which is composed of coagulation-sedimentation, media filtration, and free chlorine disinfection. Bulk water samples were collected from each treatment step over an eight-month period. qPCR targeting the universal fungal ITS gene was conducted to evaluate the fungal abundance along the treatment process, and a decreasing trend of fungal abundance was observed. Next generation sequencing targeting the ITS1 gene region was applied to investigate the fungal community dynamics. The study found the phyla Ascomycota dominated the fungal community, Basidiomycota and Zygomycota were the second and third

most abundant fungal phyla; the genera *Penicillium* and *Aspergillus* persisted through the treatment process train, with increasing relative abundance. Fungal alpha-diversity, as measured by the number of genera, decreased along the treatment process. Fungal beta-diversity, a measure of microbial community structure variation between sampling sites, was evaluated through the water treatment process. It was found that fungal community structure was significantly shifted by the media filtration step, but disinfection did not have an apparent effect on the fungal community structure. This project represents the first detailed analysis of fungal community dynamics along a conventional drinking water treatment process, demonstrating the selection effect posed by drinking water treatment process on waterborne fungi.

Chapter 4: Fungal Diversity in a Hospital Premise Plumbing System Treated with On-Site Monochloramine

Chapter 4 evaluates fungal community structure dynamics before and after secondary disinfection in a hospital premise plumbing system. Sequencing of the fungal ITS1 gene region was utilized to investigate the fungal diversity in the premise plumbing system and the potential community structure variation in response to initiation of on-site monochloramine disinfection. The results demonstrate that the genera *Penicillium*, *Aspergillus*, *Peniophora*, *Cladosporium* and *Rhodosporidium* comprised the core fungal biome of the hospital hot water system. *Penicillium* dominated the fungal community with an average relative abundance of 88.89% (± 6.37%). ITS1 sequences of fungal genera containing potential pathogens such as *Aspergillus*, *Candida*, and *Fusarium* were also detected in this study. No significant change in the fungal community structure was observed before and after the initiation of on-site monochloramine water treatment, consistent with the results of Chapter 3 that free chlorine disinfection did not clearly shift fungal

community. This work represents the first report of the effects of on-site secondary water disinfection on fungal ecology in premise plumbing system, and demonstrates the necessity of considering opportunistic fungal pathogens during the evaluation of secondary premise plumbing disinfection systems. This chapter has been published as: Ma X, Baron JL, Vikram A, Stout JE, Bibby K. Fungal Diversity and Presence of Potentially Pathogenic Fungi in a Hospital Hot Water System Treated with On-Site Monochloramine. Water Research, 2015. 71(15): p. 197-206 [37].

Chapter 5: Inactivation Kinetics of *Aspergillus* and *Penicillium* by Free Chlorine and Monochloramine in Drinking Water

Chapter 5 investigates the disinfection kinetics of *Aspergillus* and *Penicillium* by free chlorine and monochloramine. *Aspergillus fumigatus*, *Aspergillus versicolor*, and *Penicillium purpurogenum* were tested in this study. Bench-top experiments were conducted to evaluate inactivation rate of tested fungal strains when exposed to free chlorine and monochloramine. Inactivation of fungal strains was quantified using culture plate count method. The observed data were then fit to the delayed Chick-Watson model, which is a modified version of Chick-Watson model accounting for an initial lag phase of microbial cell die-off. Based on the observed data, the Ct values (product of disinfectant concentration C × contact time t) for 99.9% inactivation of the tested fungal strains ranged from 48.99 mg•min/L to 194.7 mg•min/L for free chlorine; and ranged from 90.33 mg•min/L to 531.3 mg•min/L for monochloramine. The required 99.9% inactivation Ct values for the tested fungal strains are higher than E. coli, which is commonly monitored indicator bacteria; and within the similar range for notable disinfection resistant bacteria such as *Mycobacterium* spp. and *Legionella* spp. [38-40]

Chapter 6: Summary and Conclusions

Chapter 6 summarizes the main findings and contributions of the dissertation work, including key conclusions and implications for practice.

2.0 CURRENT STATE OF KNOWLEDGE OF FUNGAL ECOLOGY IN DRINKING WATER

This work is being prepared for publication as:

Ma X, Bibby K., Fungal Ecology in Drinking Water Systems.

2.1 INTRODUCTION

Drinking water has been recognized as a non-sterile environment hosting a diverse microbiome including both bacteria and eukaryotic microorganisms, as the total elimination of microorganisms from drinking water is impractical with current technologies [1, 2]. Previous research regarding the microbiological quality of drinking water has also found the presence of fungi [3-17]. The bacterial ecology in drinking water has been extensively evaluated [18, 19]. However, research on drinking water fungi is relatively scarce, a limited number of studies have investigated the presence and diversity of fungi in drinking water, and a knowledge gap still exists regarding the fungal ecology in drinking water systems [20].

Drinking water is also an important route of transmission for opportunistic pathogens such as *Legionella pneumophila* and nontuberculous mycobacteria [21-24]. Notable fungal opportunistic pathogens, such as *Aspergillus fumigatus* and *Fusarium* spp., have been recovered

from drinking water systems [25, 26], and it has been suggested that drinking water is potentially a route of transmission for pathogenic fungi [26, 27]. Thus, understanding the drinking water fungal ecology is important for updating the opportunistic pathogen risk assessment and control [18].

In the current chapter we review the current state of knowledge of fungi in drinking water systems. As the number of studies regarding fungi in municipal treated drinking water is relatively limited, we comprehensively reviewed published studies using the Web of Science and Google Scholar focusing on fungi in municipal treated drinking water. The scope of the review is commonly recovered fungal isolates from drinking water, results from emerging culture-independent molecular techniques for studying fungal ecology, disinfection of fungi in water systems, and the potential human health significance of drinking water fungi.

2.2 COMMONLY ISOLATED FUNGAL GENERA

Culture isolation is the most frequently used method to investigate fungi in drinking water [3-10, 12-15, 20]. Previous culture-dependent studies have aimed to identify the taxonomy of fungal strains isolated from drinking water, the isolation frequency of each identified fungus (the number of positive samples/total number of samples), and the abundance of identified fungi [4-12, 25, 26, 28-30]. Among all reviewed studies regarding fungi in drinking water, twenty-four studies used culture isolation to evaluate fungal diversity in municipal drinking water or premise plumbing systems receiving municipal drinking water. The identified fungal taxa in these studies were compared and ranked based on the number of studies reported.

Previous culture-based studies have found diverse fungi in drinking water with wide ranges of isolation frequency and abundance. More than twenty different fungal genera have been isolated, and the majority of isolated fungi belong to the Ascomycota phylum [3-16, 25, 26, 28-37]. Among fungal taxa previously isolated by culture, six fungal genera were found in more than half of the reviewed studies that focused on fungal diversity in municipal treated drinking water: *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma* [3-15, 25, 26, 28, 29, 31, 36]. Fungal genera isolated by more than two studies include *Alternaria*, *Paecilomyces*, *Phialophora*, *Phoma*, *Aureobasidium*, *Candida*, *Mucor*, *Chaetomium*, *Rhodotorula*, *Beauveria*, and *Verticillium* [4-8, 11-16, 28, 32, 33, 35-37] (Table 2.1).

Published data shows that the isolation frequency of *Penicillium* spp. among drinking water samples ranged from 3.03% to 70.2%, with abundance ranging from 2.6 CFU/L to 288 CFU/L [4-12, 28, 29]. *Aspergillus* spp. has been frequently isolated from drinking water with published isolation frequency ranged from 6% to 42.1% and abundance ranging from 1.9 CFU/L to 101.3 CFU/L [4-11, 25, 26, 28, 29]. The isolation frequency of *Cladosporium* spp. was found in 1.6% to 74.6% of samples, with published abundance ranging from 3 CFU/L to 140 CFU/L [5, 7-11, 28]. *Fusarium* spp. was recovered from drinking water with published isolation frequencies from 0.6% to 57% and abundance ranging from 1 CFU/L to 105 CFU/L [4, 7, 8, 11, 26, 28, 30]. *Acremonium* spp. was found in drinking water systems with reported isolation frequency ranging from 0.4% to 25.6%, and the abundance of *Acremonium* spp. was reported to range from 2 CFU/L to 260 CFU/L [4, 5, 7, 8, 10, 28, 29]. *Trichoderma* spp. was reported with a range of isolation frequency from 0.4% to 36.9%, and abundance range from 2.2 CFU/L to 120 CFU/L [4, 5, 7, 8, 11, 16, 28]. Figure 2.1 and 2.2 show boxplots of isolation frequency and abundance for these genera commonly found in municipal drinking water summarized from published data.

Table 2.1 List of fungal genera isolated by more than two studies

Isolated fungal phylum	Isolated fungal genera	References
Ascomycota	*Penicillium*	[3-12, 14-16, 28, 29, 31-36]
	Aspergillus	[3-12, 14-16, 25, 26, 28, 29, 31, 32, 35, 36]
	Cladosporium	[3, 5, 7-12, 14-16, 28, 31, 32, 34-37]
	Acremonium	[4, 6-8, 10, 12-16, 28, 29, 32-36]
	Fusarium	[3, 4, 7, 8, 11-16, 26, 28, 30, 33, 35]
	Trichoderma	[3-5, 7, 8, 11, 14-16, 28, 31, 35]
	Alternaria	[4, 5, 7, 8, 12, 14, 16, 32, 33, 36, 37]
	Paecilomyces	[4, 7, 8, 12-16, 32, 35]
	Phoma	[7, 10, 15, 16, 32, 33]
	Phialophora	[3, 10, 15, 34, 36, 37]
	Candida	[32, 33, 35]
	Aureobasidium	[11, 28, 32, 35]
	Verticillium	[6, 15]
	Beauveria	[28, 33]
	Chaetomium	[15, 36]
Basidiomycota	*Rhodotorula*	[10, 32, 35]
Zygomycota	*Mucor*	[4, 11, 16, 32]

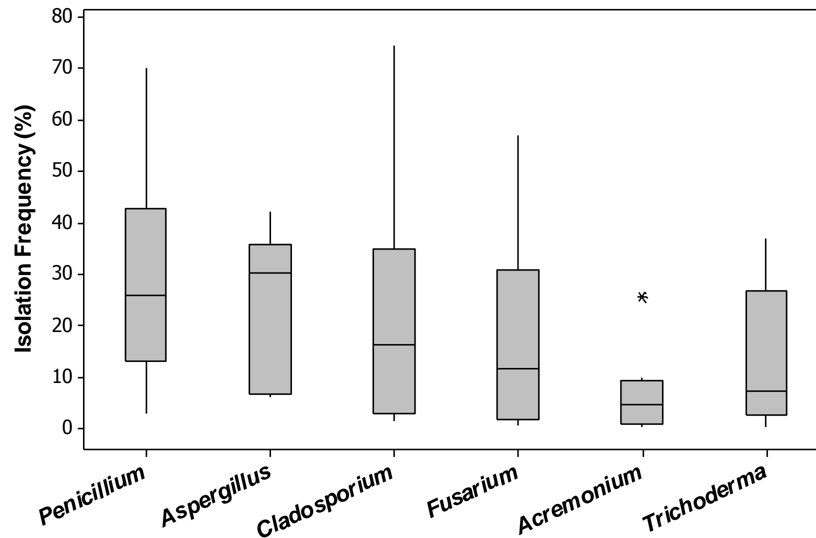

Figure 2.1 Isolation frequency (= number of positive samples/total number of samples) of the top six most commonly isolated fungal genera; data summarized from published research, number of studies with reported isolation frequency was 14, 12, 10, 9, 8, and 8, respectively for *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma* [4-12, 16, 25, 26, 28-30]

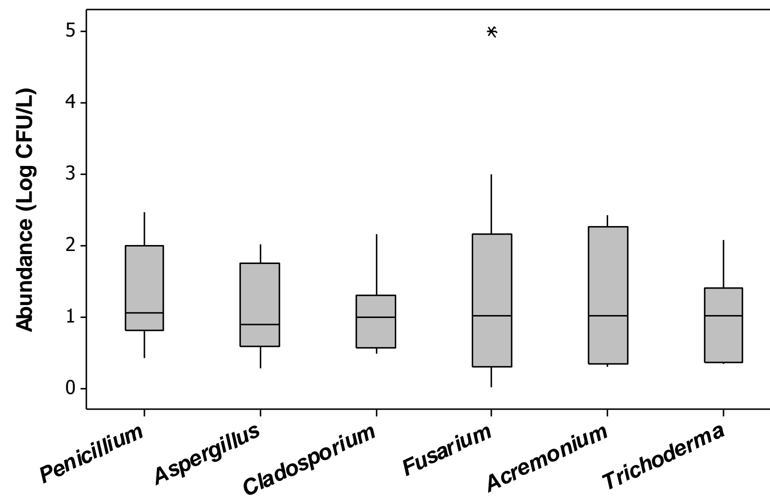

Figure 2.2 Abundance of the top six most commonly isolated fungal genera; data summarized from published research, number of studies with reported isolation frequency was 9, 10, 5, 7, 4, and 5, respectively for *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma* [4-12, 16, 25, 26, 28-30]

Biofilms, agglomerated microbial growth attached to surfaces, can be widely found in drinking water systems and have been suggested as one important source of planktonic phase microorganisms [21, 38]. Biofilms also provide protection against water disinfection enabling better survival of microorganisms in drinking water [18, 21, 39]. Fungal hyphae and spores have previously been detected in drinking water distribution system biofilm samples using in-situ Calcofluor white M2R staining, indicating that biofilms also provide a survival niche to fungi, in addition to bacteria and other microorganisms [40, 41]. A previous study evaluated the abundance and diversity of fungi in biofilm samples collected from a drinking water distribution system and determined that the total fungal abundance ranged from 8.9 CFU/cm2 to 31.8 CFU/cm2 in the biofilm [32]. This study also found *Penicillium* and *Aspergillus* represented the most abundant fungal genera in the biofilm [32]. Evidence shows drinking water system biofilms

also provides ideal niche for free living amoeba (FLA), which harbor and promote survival of pathogenic bacteria, such as *Legionella* spp., in drinking water [42]. Similar interaction between common waterborne FLA and fungi were also observed by recent research, demonstrating that survival of *Aspergillus fumigatus*, *Candida* spp., and *Fusarium oxysporum* were enhanced in tap water when co-cultured with FLA [43-45].

As fungi are typically dilute in drinking water, fungal isolation requires the culture of concentrated water samples on nutrient media. Membrane filtration has been commonly used for fungal concentration [3, 25, 26]. Centrifugation has also been used for concentrating fungal cells in water samples, but has been suggested to be limited when processing high-volume samples [26, 31]. Direct plating of 0.1 mL to 1 mL water samples on nutrient media has also been used for fungal isolation and enumeration [31]. Thus, isolation of fungi has been performed using variable volumes of water in individual studies, resulting in different detection limits, and subsequently limiting cross-study comparisons [20].

The selection of cultivation media also varies among studies. Commonly used media includes malt extract agar [3, 17], sabroud dextrose agar [25], potato dextrose agar [6], and Czapek-Dox agar [6]. Chloramphenicol was often used as the antibiotic spiked in isolation media to inhibit bacterial growth [3, 6, 25]. Dichloran was also used as an additive to prevent overgrowth of fast-growing fungal species [28]. A previous study comparing different culture media for fungal isolation from drinking water has demonstrated that sabroud dextrose agar recovered more species from *Penicillium* spp., and *Aspergillus* spp.; while media such as half-strength corn meal agar recovered more species from other genera [31]. Therefore, it is impossible to isolate all fungal species using only one or a few combinations of culture media, limiting the output when using culture isolation to evaluate fungal diversity in drinking water

[20, 31]. Also, culture techniques are challenged for detecting viable but nonculturable microorganisms, including fungi, in drinking water [18, 46]. Given these limitations of culture techniques, additional methods are necessary to reach a holistic understanding of fungal ecology in drinking water. Research opportunities exist regarding the evaluation of fungal diversity in drinking water and to identify the universal core fungal community members in drinking water, as well as to determine a representative range of fungal abundance in drinking water.

2.3 EMERGING CULTURE-INDEPENDENT TOOLS FOR INVESTIGATING FUNGAL ECOLOGY

Drinking water contains highly complex microbial communities, of which the majority are viable but nonculturable [1, 2, 18]. Culture isolation provides confirmation of viability of fungi in drinking water, but has a limited ability to reveal diversity and community structure change among different stages of drinking water systems. High-throughput next generation sequencing of fungal gene markers supplements this limitation, providing a more detailed analysis of fungal diversity and community structure change in correlation with environment [47].

Recent studies have used culture-independent next generation sequencing of the 18S rRNA gene to assess eukaryotic diversity, including fungi, in drinking water [22, 48-50]. Sequencing of the 18S rRNA gene was applied to investigate fungal diversity in drinking water used in a dental unit waterline [49]. This study showed the fungal community was dominated by fungal phyla Ascomycota and Basidiomycota; on the genus level *Mrakia* spp. and *Candida* spp. were found to be the dominant genera [49]. Another study utilized 18S rRNA sequencing to

investigate the fungal community in both bulk water and the biofilm of a drinking water distribution system, showing that *Aspergillus* spp., *Rhexocercosporidium* spp., and *Plectosphaerella* spp. were detected in all samples [48]. The bulk water fungal community structure and biofilm fungal community were also observed to be distinct [48]. A study utilizing 16S rRNA and 18S rRNA sequencing to evaluate both the bacterial and eukaryotic community along a drinking water treatment process also found Ascomycota as the dominant fungal phylum in drinking water, and a change in eukaryotic community structure along the drinking water treatment process was found [50].

The internal transcribed spacer (ITS) gene region, which is more hypervariable in fungi than the 18S rRNA gene, has been proposed as a universal gene marker for fungi [51]. Culture-independent ITS gene sequencing has been applied to assess fungal diversity in soil and air [52-54], but research utilizing ITS sequencing to assess fungal ecology in drinking water is still limited. A recent study, included in this dissertation as Chapter 4, evaluated fungal ecology in a hospital water system using fungal ITS sequencing, and demonstrated the fungal community was dominated by *Penicillium* spp., with *Aspergillus* spp. also frequently detected [55]. In addition, the total fungal community structure did not demonstrate a significant change in response to addition of monochloramine as supplemental disinfection to this water system [55].

Drinking water systems from treatment, distribution, to premise (building) plumbing are a highly complex environment. Each stage of the drinking water system provides a unique survival niche for microorganisms, potentially shaping the microbial community structure [18]. Recent studies using culture-independent approach demonstrated relations between fungal community structure and engineering factors of drinking water system, such as centralized water treatment process and premise plumbing disinfection [50, 55]. However, research regarding

fungal community dynamics in drinking water is still limited. With the recent development of high-throughput next generation sequencing, research opportunities exist to evaluate the fungal communities within drinking water systems. It should be noted that the current culture-independent sequencing methods are inherently limited by the lack of clear differentiation between viable and non-viable cells [18, 47]; further development of viability-specific sequencing approaches are needed [18].

2.4 DISINFECTION OF FUNGI IN DRINKING WATER

Disinfection, generally the last step of drinking water treatment, is critical to minimize pathogens and infectious risks from drinking water by maintaining a disinfectant residual in treated water [18, 56]. Knowledge of microbial inactivation kinetics is important for quantifying pathogen inactivation and the estimation of microbial risks from drinking water [57]. The resistance of fungi to water disinfectants has only been studied to a limited degree. An early study compared the free chlorine sensitivity of *Aspergillus fumigatus*, *Aspergillus niger*, *Cladosporium* spp., *Penicillium oxalicum*, and coliform bacteria [58]. The study found that the evaluated fungi survived up to 60 minutes exposure to free chlorine residual level ranged from 0.64 mg/L to 6.7 mg/L; where no survival of coliform bacteria was observed [58]. However, disinfection kinetics and disinfection dose for certain $\log_{10}$ inactivation levels following Ct (concentration-time) concept were not measured in this study because of limited data [58]. A previous study also compared the survival of *Aspergillus calidoustus*, *Penicillium spinulosum*, *Trichoderma viride*, and *Fusarium solani* in water when exposed to commonly used water disinfectants,

demonstrating that the resistance of fungi to disinfection is species dependent [59]. A recent study evaluated free chlorine inactivation of waterborne fungi in post sedimentation water collected from a drinking water treatment plant, and found 60 mg•min/L of Ct (product of concentration C $\times$ contact time t) was needed to inactivate 80% fungi in the settled surface water [60]. However, the Ct values in this study were estimated based on aimed initial disinfectant concentration but not measured real-time disinfectant concentration, therefore more detailed evaluations of fungal disinfection kinetics following Ct concept are still needed [60]. Inactivation of *Aspergillus* spp. in water by ultraviolet (UV) radiation was also previously evaluated; 4-$\log_{10}$ inactivation for *A. fumigatus*, *A. niger*, and *A. flavus* were achieved at UV doses of 12.5 mJ/cm^2, 16.6 mJ/cm^2, and 20.8 mJ/cm^2, respectively, and the required UV doses were higher than the dose for 4-$\log_{10}$ inactivation of *Legionella pneumophila*, which was 11 mJ/cm^2 [61]. Currently, disinfection of fungi in drinking water has only been investigated by a limited number of studies [58-61], and detailed evaluation of disinfection kinetics for many fungi is still lacking.

2.5 POTENTIAL HUMAN HEALTH SIGNIFICANCE OF DRINKING WATER FUNGI

Among the most commonly isolated drinking water fungi, *Aspergillus* spp. and *Fusarium* spp. have been recognized as prevalent opportunistic pathogens among immunocompromised patients [62-64]. Studies reviewing invasive fungal infection data have showed that *Aspergillus* spp. is the second most prevalent cause of opportunistic fungal infections after *Candida* spp., accounting for 29% of infections among transplant patients [62, 65]; and published data indicate

the infection has a high mortality rate of 62% to higher than 85% [62, 63, 65-68]. *Aspergillus* infections are most frequently caused by *Aspergillus fumigatus*, followed by *A. flavus*, *A. niger*, *A. terreus*, and *A. nidulans* [62, 63, 68]. Meanwhile, the most commonly found *Aspergillus* species from drinking water were *A. niger* [3, 5-8, 11, 25, 28, 35], *A. fumigatus* [5-9, 25, 26, 28], *A. terreus* [3, 5, 6, 10, 25, 26, 35], and *A. flavus* [3, 5, 7, 8, 11, 25, 26]. Other species less commonly recovered include *A. ustus*, *A. clavatus*, *A. penicillioides*, *A. carbonarius*, *A. glaucus*, *A. nidulans*, *A. ostianus*, *A. parasiticus*, *A. restrictus*, *A. ochraceus*, *A. sydowii*, and *A. versicolor* [3, 5, 6, 8, 28, 31, 35]. The spectrum of *Aspergillus* species in drinking water includes pathogenic species, and hospital drinking water has previously been suggested to potentially be one of the transmission paths for opportunistic *Aspergillus* infections [25]. Genetic similarity between *A. fumigatus* strain isolated from patients and *A. fumigatus* strain isolated from hospital tap water samples were previously revealed using an amplified fragment length polymorphism (AFLP), suggesting the possibility that patients might had contracted waterborne *A. fumigatus* [69].

The *Fusarium* genus is another important opportunistic fungal pathogen, and common species causing infections include *F. solani*, *F. oxysporum*, and *F. moniliforme* [62, 70]. *Fusarium* spp. were frequently recovered from drinking water [4, 7, 8, 11, 26, 28, 30], and *F. solani* and *F. oxysporum* were the most frequently identified species. Other less commonly identified species included *F. dimerum* and *F. sporotrichioides* [4, 11, 26, 28]. Studies also demonstrated genetic similarity between clinical *F. oxysporum* isolates and *F. oxysporum* isolates from a drinking water supply using a common translation elongation factor 1α gene (TEF1) and ribosomal intergentic spacer (IGS) [71, 72]. This genetic match further supports the hypothesis that drinking water might be a source of *Fusarium* infection [26, 71, 72].

Acremonium spp. and *Trichoderma* spp., which were also commonly isolated from drinking water, were not initially recognized as major opportunistic fungal pathogens; however, more recent research has suggested *Acremonium* spp. and *Trichoderma* spp. as emerging opportunistic pathogens because of reported cases of infection [73-76].

Previous research had recovered diverse fungal species from drinking water, and the spectrum of waterborne fungi contains multiple opportunistic fungal pathogens (*Aspergillus* spp., *Fusarium* spp., *Acremonium* spp., and *Trichoderma* spp.). As previously suggested, drinking water has potential to be one route of transmission for these opportunistic fungal pathogens [26]. Genetic similarity between tap water isolated fungal strains and infected patient isolated strains has been revealed and suggests drinking water to be an environmental source of fungal pathogens [26, 69, 71, 72]. However, the mechanisms regarding how patients contracted these fungal pathogens from water have not been demonstrated. The hypothesis has been raised that drinking water fungi may contribute to indoor airborne fungi through aerosolization of tap water, and subsequently cause secondary-inhalation [26, 77]. Further research is needed to fill this knowledge gap and will potentially benefit updated risk assessment of waterborne pathogen and control of nosocomial fungal infection.

2.6 SUMMARY AND CONCLUSIONS

Published research shows drinking water contains diverse fungal species, and *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma* compose the most commonly isolated fungal genera from drinking water. The spectrum of drinking water fungi

also contains several notable opportunistic fungal pathogens, such as *Aspergillus fumigatus* and *Fusarium* spp. However, studies of drinking water fungi are limited. A holistic understanding of fungal diversity and community structure change within the drinking water system is still lacking, due to both the limited number of studies and the methodological limitation of culture-dependent methods. Next-generation sequencing of fungal marker genes enables a more detailed analysis of fungal ecology in drinking water systems. A major knowledge gap exists regarding fungal community structure dynamics in drinking water systems and the potential relation with engineering factors, such as centralized drinking water treatment process and addition of disinfectants to premise plumbing systems. Further research is also needed to evaluate the disinfection kinetics of fungi in drinking water.

3.0 DRINKING WATER FUNGAL COMMUNITY STRUCTURE SHAPED BY CENTRALIZED DRINKING WATER TREATMENT OPERATIONS

This work is being prepared for publication as:

Ma, X., Vikram, A., Casson, L., Bibby, K., Centralized Drinking Water Treatment Operations Shape Bacterial and Fungal Community Structure

3.1 INTRODUCTION

Centralized drinking water treatment processes are designed as a barrier to remove potential pathogens from the raw water and greatly reduced the burden of waterborne infectious diseases [1, 2]. Although drinking water treatment processes greatly reduce the microbial abundance, the treated drinking water is far from a sterile environment and carries diverse microbial communities [1, 3]. Studies have estimated the total bacterial cell abundance in drinking water ranges from 10^3 to 10^5 cells/mL [3-5]. Microbial eukaryotes such as fungi and free-living amoeba have also been frequently isolated from drinking water [6-8]. Within this post-treatment drinking water microbiome, opportunistic pathogens have emerged as a public health issue, posing the greatest infectious disease risk associated with drinking water in the industrialized

countries [3, 9]. Members of the drinking water microbiome also have potential to cause other negative impacts such as nitrification and biocorrosion [1, 3].

Total elimination of microorganisms from drinking water is impossible with current technologies [9, 10]. On the other hand, recent studies have demonstrated that the drinking water treatment process alters microbial community structure [10, 11], suggesting a potentially beneficial opportunity to manage drinking water microbial community structure [3, 9]. Only a limited number of studies have investigated the bacterial community structure variations within drinking water treatment processes [10-12]. A notable previous study demonstrated that bacteria colonizing filter media governed the downstream bacterial community structure, suggesting this process to be a primary driver of downstream microbial community structure [10].

Holistic evaluation of fungal community structure variations within drinking water treatment process has not been conducted, despite the fact that drinking water microbiome also contains diverse fungal species, including potential opportunistic pathogens such as *Aspergillus* spp. and *Fusarium* spp. [13-15]. A knowledge gap still exists regarding the dynamics of microbial community structure along the treatment process and operational factors that affect the microbial community structure. Further studies are still needed to generalize a universal pattern of drinking water microbial community dynamics considering more groups of microorganisms and more engineered processes [3].

The present study evaluates the fungal and bacterial community along a conventional drinking water treatment process which uses media filtration and free chlorine disinfection. The goal of this study was to investigate the influence of drinking water unit operations, including media filtration and disinfection, on fungal community structure in the context of centralized water treatment. Bacterial community structure was also evaluated to enable a holistic

comparison of observed results to previous studies. Next generation sequencing targeting the ITS1 gene region was used to characterize the fungal community, while 16S rRNA sequencing was used to characterize the bacterial community. Fungal and bacterial abundance, as well as the presence of *Acanthamoeba* spp. and *Aspergillus fumigatus* were evaluated using PCR. Culture enumeration was also conducted to evaluate the culturable heterotrophic bacterial abundance and filamentous fungal abundance.

3.2 MATERIALS AND METHODS

3.2.1 Sampling Site

An anonymous surface water U.S. drinking water treatment plant was selected as the study site. The treatment process is outlined in Figure 3.1. The treatment plant uses conventional surface water treatment process unit operations similar to many treatment plants around the U.S. River water is pumped into the plant as raw water, and ferric chloride and cationic polymer (approximately 25 mg/L and 1 mg/L, respectively) are added for coagulation. The water then passes through the clarifier for sedimentation, and is settled for an additional 24 hours in a secondary sedimentation basin. The settled water passes through a media filter composed of coal, sand, and support gravel. Free chlorine disinfection using sodium hypochlorite is the last step of treatment, and a free chlorine residual ranging from 0.4 to 1.0 mg/L was maintained during the study period.

3.2.2 Sample Collection

Bulk water samples were collected from four locations at the treatment plant (Figure 3.1): (1) raw water at the pump to the treatment plant; (2) post-sedimentation water, the effluent from the secondary sedimentation basin; (3) post-filtration water, the effluent from the rapid sand filter; (4) post-disinfection water, the effluent from the disinfection clear well. Eight duplicate monthly water samples were collected from these four locations on January, February, March, April, May, June, July, and September 2014. Samples were collected in duplicate using 1-L sterile Nalgene polypropylene sampling bottle without preservative.

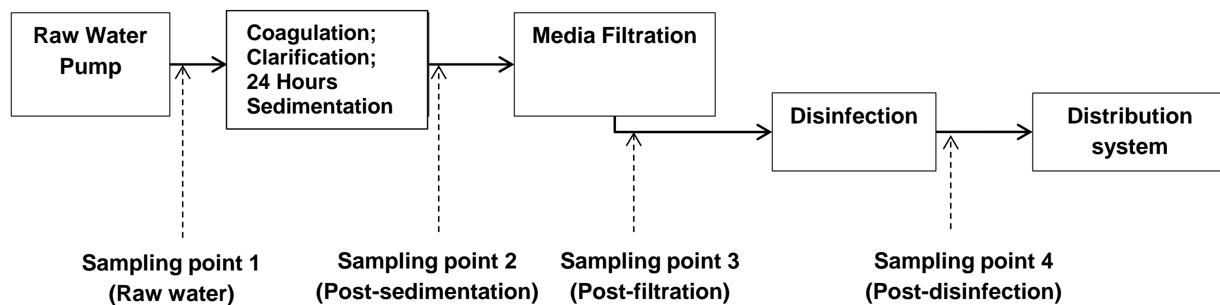

Figure 3.1 Sampling locations along the surface water treatment unit processes; post-sedimentation samples represent water after 24 hours sedimentation

Water samples were immediately transported on ice to the laboratory. Upon arrival, 500–1900 ml of each water sample was filtered through 0.2 µm Supor® 200 Polyethersulfone membranes (Pall Corporation) housed in sterile Nalgene analytical filter funnels (Thermo Scientific; Fisher) within a class II biological safety cabinet. The filter membrane with biomass

was then stored in a sterile 10 mL tube at -80◦C until DNA extraction. The remaining subset of the water samples were measured for conductivity (Accument conductivity meter, Fisher Scientific), pH (Russell RL 060P pH meter, Thermo Scientific), and free chlorine (Hach colorimeter, Hach) following manufacturer's protocols.

Two additional weekly sample sets from the described sampling locations were collected during June and July 2014. These additional samples were analyzed for heterotrophic plate count (HPC) and total filamentous fungal colony count to confirm with qPCR results of the current study. Detailed methods for HPC and total filamentous fungal colony count analysis are listed in Appendix A.

3.2.3 DNA Extraction, PCR, and Sequencing

Frozen filter membranes with biomass collected were thawed at 4ºC, and subjected to DNA extraction using a RapidWater DNA Isolation Kit (MO BIO Laboratories, Inc., CA) following the manufacturer's protocol. All duplicate samples were subjected to PCR targeting the fungal ITS1 gene region using fungal ITS gene specific primers ITS1F and ITS2 modified for Illumina multiplex sequencing as previously described [16]. One replicate for each sample were subjected to PCR of 16S rRNA gene using previously described primers 515F and 806R [17]. A detailed description of PCR conditions for ITS1 and 16S rRNA PCR can be found in Appendix A. DNA extraction negative controls with blank filter membrane were included to ensure no contamination introduced by DNA extraction kit and no-template PCR negative controls were included in all PCR runs to ensure reagents and equipment were not contaminated. All controls were negative for contamination. Two pure culture fungal DNA sample, *Penicillium*

chrysogenum (CAES PC-1) and *Aspergillus fumigatus* (ATCC 34506), were included in the ITS1 PCR as positive controls. The PCR products of the two pure culture fungal DNA were included in the ITS1 library to confirm the accuracy of fungal taxonomy classification.

PCR products were then purified with Agencourt AMPure XP magnetic beads (Beckman Coulter), purity confirmed by gel visualization, the purified PCR products were then quantified by Qubit 2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Invitrogen), and normalized and pooled based on equal molarity to construct fungal ITS and bacterial 16S rRNA amplicon libraries. One additional purification step was performed for each pooled library with Agencourt AMPure XP magnetic beads. Then the ITS and 16S rRNA libraries were sequenced on Illumina Miseq Sequencer (Illumina) in two separate runs.

3.2.4 Bioinformatics

Raw sequence data of the 16S rRNA library was demultiplexed and trimmed based on a quality score of 20 using QIIME 1.8.0 [18]. Sequences after trimming were grouped into operational taxonomic units (OTUs) at 97% similarity and classified from phylum to genus level using closed reference OTU picking strategy and Greengenes 13.5 as reference database [19]; sequences classified as chloroplast were removed. As previous study demonstrated that grouping fungal ITS sequencing reads into OTUs limits taxonomy classification accuracy of fungal community [20], raw sequencing data of the ITS1 library was trimmed and demultiplexed based on a quality score of 20 using QIIME 1.8.0 [18] and compared directly against reference ITS database [21] using BLAST ver. 2.2.19 [22]. The BLASTn output was further sorted using FHiTINGs [23].

Unweighted and weighted UniFrac matrices were calculated to evaluate the degree of bacterial community structure similarities between each sample [24]. As UniFrac, which relies on global alignments, is infeasible for the ITS gene region [25], Jaccard and Bray-Curtis matrices were calculated using the R package Ecodist based on fungal taxonomy classification at the genus level [26, 27]. Principal coordinate analysis (PCoA) was performed to evaluate community structure variations between each sampling group and pairwise ADONIS analysis (permutational multivariate analysis of variance) [28] implemented within QIIME [18, 29] was used to further assess the statistical significance of difference in community structure among sampling groups. The raw sequencing data are publically available on MG-RAST under accession number of 4723108.3 and 4723110.3.

3.2.5 qPCR of Total Fungi, Total Bacteria, *Acanthamoeba* spp., and PCR of *Aspergillus fumigatus*

qPCR based on SYBR Green was conducted to quantify the abundance of total fungi, total bacteria, and *Acanthamoeba* spp. qPCR targeting total fungi was conducted with previously described fungal universal primers ITS1FI2 and ITS2 targeting ITS1 region of rDNA [30, 31]. qPCR targeting total bacteria was carried out by using previously described primers targeting the universal bacterial 16S rRNA gene region [32]. qPCR for *Acanthamoeba* spp. quantification was performed by using previously described primers AcantF900 and AcantR1100 [33]. Detailed qPCR conditions can be found in Appendix A. No-template qPCR negative controls were included in all qPCR runs to ensure reagents and equipment were not contaminated

A standard curve for fungal ITS qPCR was constructed using serial dilutions of a cloned TOPO plasmid with *Aspergillus fumigatus* (ATCC 34506) ITS1 amplicon inserted. The standard curve for bacterial 16S rRNA qPCR was constructed directly using serial dilution of genomic DNA extracted from pure culture of *Pseudomonas fluorescens* (ATCC 13525). Standard curves of *Acanthamoeba* spp. qPCR was constructed by using cloned TOPO plasmid containing *Acanthamoeba castellanii* (ATCC 30010D) 18S rRNA amplicon. Plasmids for fungal and *Acanthamoeba* spp. qPCR were cloned using a TOPO TA cloning kit (Invitrogen). Insertion of a single copy of amplicon was verified by Sanger sequencing conducted at Genomic Research Core, University of Pittsburgh. Inhibition testing was performed by spiking sample DNA into known amount of standard; no inhibition of qPCR was observed. Previous studies have shown an average genome size of 6,745,279 bp for *P. fluorescens* [34, 35]; an average 55 ITS copies per genome of *A. fumigatus* [36]; and 600 18S copies per genome of *A. castellanii* [37]. Thus qPCR results in the current study were presented as genome equivalents/mL accordingly. Based on the standard curves and volume of sample filtered, the bacterial qPCR lower detection limit in water sample was 0.54 *P. fluorescens* genome equivalents/mL; the lower detection limit of fungal qPCR was 0.22 *A. fumigatus* genome equivalents/mL (12.1 ITS copies/mL); and the lower detection limit of *Acanthamoeba* spp. was 0.002 *A. castellanii* genome equivalents/mL (1.4 18S copy/mL).

The presence of *Aspergillus fumigatus* was screened using previously described PCR primers targeting the *A. fumigatus* alkaline protease gene [38, 39]. Positive controls using genomic DNA extracted from pure *A. fumigatus* culture (ATCC 34506) as template, and no-template negative controls were included. PCR results were analyzed using a 1% agarose gel. Detailed PCR conditions can be found in Appendix A.

3.3 RESULTS AND DISCUSSION

3.3.1 Sampling and Analysis Overview

In total, 64 samples (duplicate samples of four locations over eight months) were collected from a single anonymous drinking water treatment plant. All duplicate samples were subjected to universal fungal ITS1 PCR, with 61 out of 64 samples resulting in positive amplification. The sequencing results of the two positive ITS1 amplicon controls indicated high accuracy to genus level fungal classification as 99.9% of sequences of *Aspergillus fumigatus* ITS1 PCR products were assigned to *Aspergillus* spp. and 99.9% of sequences of *Penicillium chrysogenum* ITS1 PCR products were accurately assigned to Penicillium spp. These results are consistent with previous observations of ITS1 classification [40]. No significant community structure variation between ITS1 sequencing results of replicate samples was observed (ADONIS p-values based on Jaccard and Bray-Curtis matrices > 0.05). Additionally, previous research demonstrated high reproducibility of 16S rRNA sequencing results between replicate samples [41] and technical replicates [16]. Thus, one replicate of each sample (32 samples) was subjected to universal 16S rRNA PCR, with all resulting in positive amplification. In total, 446,902 ITS1 sequence reads and 548,098 16S rRNA sequence reads were used for taxonomic classification after demultiplexing and quality trimming. Statistics of trimmed sequences are listed in Appendix A-Table A1. All negative controls were PCR negative indicating no contamination of reagents and equipment.

3.3.2 qPCR of Total Fungi, Total Bacteria, *Acanthamoeba* spp., and PCR of *Aspergillus fumigatus*

The average fungal abundance was 3.4±0.5 log genome equivalents/mL in raw water, 1.6±0.5 log genome equivalents/mL post-sedimentation, 0.9±0.4 log genome equivalents/mL post-filtration, and 0.2±0.4 log genome equivalents/mL post-disinfection (Table 3.1). A significant decrease in fungal abundance was observed post-sedimentation and post-filtration (p-values < 0.05). The average post-disinfection fungal abundance was lower than post-filtration, yet without a statistically significant change ($p > 0.05$). The average total bacterial abundance was 5.5±0.4 log genome equivalents/mL in raw water, 3.4±0.4 log genome equivalents/mL post-sedimentation, 2.9±0.3 log genome equivalents/mL post-filtration, and 1.2±0.3 log genome equivalents/mL post-disinfection (Table 3.1). Bacterial abundance significantly decreased after each unit operation (p-values < 0.05). The inability to discriminate DNA from viable cells and dead cells by the applied molecular methods may inflate observed fungal and bacterial abundance, especially in the post disinfection samples. To confirm with the trend of the observed results, we collected additional samples and analyzed culturable heterotrophic bacteria and filamentous fungi. The culture analysis showed similar decreasing trend through the treatment process train for both heterotrophic plate count and filamentous fungal colony count (Table 3.1). Both the qPCR and subsequent culturing results suggest the treatment process reduced bacterial and fungal abundance, agreeing with previous culture-based studies [42, 43].

As *Acanthamoeba* spp. is an important human pathogen [44] and may host other opportunistic pathogens such as *Legionella* spp. [45, 46], qPCR was conducted to evaluate the presence and abundance of *Acanthamoeba* spp. Six out of eight raw water samples and one out

of eight post filtration samples had detectable level of *Acanthamoeba* spp., with average abundances of -1.36±1.61 log genome equivalents/mL and -1.51 log genome equivalents/mL, respectively. None of the post sedimentation and post disinfection samples had detectable *Acanthamoeba* spp. Previous studies demonstrated the presence of *Acanthamoeba* spp. in treated drinking water [8, 47] and isolation of *Acanthamoeba* spp. from post filtration water samples of water treatment plant was also previously reported [48]. Our results indicated the presence of *Acanthamoeba* spp. in raw water and post filtration water, in agreement with previous research. Further study investigating the association of *Acanthamoeba* spp. with filter media would be helpful for understanding the role of media filtration in *Acanthamoeba* spp. survival during water treatment. Similarly, as *Aspergillus fumigatus* is among the most notable human fungal pathogens and has previously been isolated from drinking water [14], we conducted PCR targeting *A. fumigatus*. No sample had detectable *A. fumigatus*.

3.3.3 Microbial Diversity through the Water Treatment Processes

We applied both alpha- and beta-diversity measures to explore the influence of drinking water unit operations on the bacterial and fungal communities. Beta-diversity is a measure of microbial community structural variation between sampling sites. A dissimilarly index approach was used to evaluate the microbial beta diversity through the water treatment process.

The Jaccard matrix was calculated based on presence/absence of each fungal genera, since UniFrac, a commonly used dissimilarity matrix for bacterial beta-diversity evaluation, is not feasible for the fungal ITS gene region [25]. The mean dissimilarity level of fungal community across water treatment unit operations was statistically significant higher than the

overall average dissimilarity level across all samples and the mean dissimilarity level across different months (p-values < 0.05) (Figure 3.2-A). It indicates the fungal community structure change is mainly driven by water treatment unit processes. The PCoA plot based on the Jaccard matrix indicated fungal community structure shifted following sedimentation and media filtration. Post-disinfection samples clustered strongly with post-filtration samples, indicating that the fungal community structure in post-disinfection samples was similar to post-filtration samples, suggesting no further community structural variation occurred following disinfection (Figure 3.3-A). The pairwise ADONIS based on the Jaccard matrix further indicated that the fungal community structure significantly shifted after sedimentation and media filtration (p-values < 0.05, Appendix A Table A2), but no significant change occurred post disinfection (p > 0.05, Appendix A Table A2). PCoA and ADONIS based on the Bray-Curtis matrix, accounting for both presence/absence and relative abundance of each fungal taxa, demonstrated the same pattern of community structural variation that media filtration was the last treatment step that significantly shifted fungal community structure (Appendix A Figure A1-A, Appendix A Table A2).

For the bacterial community, the mean community dissimilarity level across water treatment unit operations was statistically significant higher than the overall average dissimilarity level across all samples and the mean dissimilarity level across different months (p-values < 0.05) (Figure 3.2-B). It indicates the bacterial community structure change is mainly driven by water treatment unit processes, similar to fungal community. the PCoA plot based on unweighted UniFrac, a dissimilarity matrix accounting for presence/absence and phylogenetic relationship between each bacterial taxa, indicated samples clustered within each sampling site and suggested bacterial community structure shifted following each unit operation (Figure 3.3-B). The

statistical significance of the community structure shift following each unit operation was further confirmed with pairwise ADONIS analysis (p-values < 0.05, Appendix A Table A3). PCoA and ADONIS based on weighted UniFrac, a dissimilarity matrix similar to unweighted UniFrac but also accounting for relative abundance of each taxa, demonstrated the same pattern of community structure variation, except that the community structural variation between post-sedimentation and post-filtration samples was not statistically significant (Appendix A Figure A1-B, Appendix A Table A3).

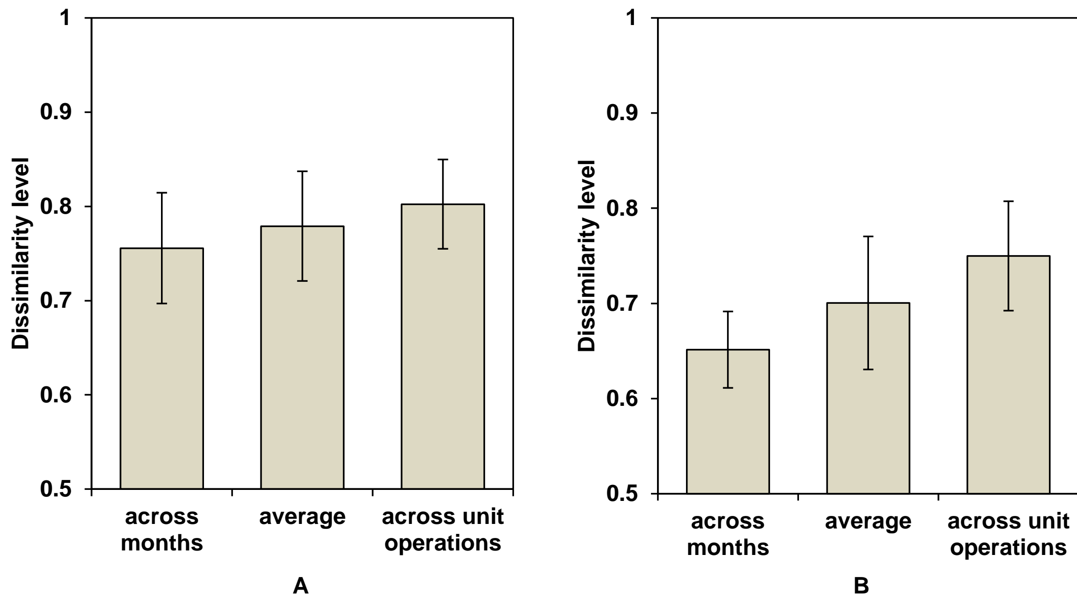

Figure 3.2 Mean community dissimilarity level of (A) fungal community and (B) bacterial community; higher dissimilarity level indicates less similar community structure

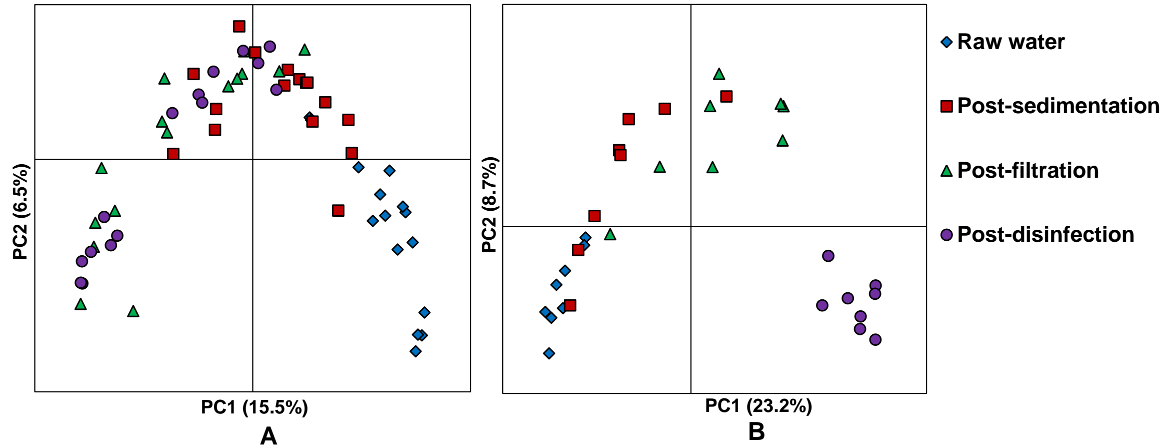

Figure 3.3 PCoA plots based of (A) fungal Jaccard dissimilarity matrix and (B) bacterial unweighted UniFrac dissimilarity matrix; each point in the figure represents one sample, points clustered closer indicate more similar community structure; post-sedimentation samples represent water after 24 hours sedimentation

We also explored alpha-diversity variation through the treatment processes via examination of community richness (total number of taxonomic units). The trend in richness for both bacterial and fungal communities was similar to the trend of bacterial and fungal abundance through the treatment process (Table 3.1). The number of bacterial OTUs significantly decreased through each step of the treatment process (p-values < 0.05) (Table 3.1). The number of fungal genera significantly decreased post-sedimentation and post-filtration (p-values < 0.05), but no significant decrease occurred post disinfection ($p > 0.05$) (Table 3.1). The finding that bacterial species richness decreased through the water treatment process agrees with previous study [11]. These results also identify a similar trend for fungal richness but no change was found post-disinfection. The observed trend in bacterial and fungal community richness also correlates with the observed trend for beta-diversity.

Table 3.1 Bacterial and fungal diversity and abundance and water quality parameters. Detection limits were -0.27 log genome equivalent/mL for bacterial qPCR and -0.66 log genome equivalent/mL for fungi.

	Raw water	Post- sedimentation*	Post-filtration	Post-disinfection
Fungal community				
Total number of genera	980	585	315	294
Average number of genera per sample	396.5±173.4	188.9±60.8	91.1±51.5	91.5±28.6
Average fungal abundance (log genome equivalent/mL)	3.4±0.5	1.6±0.5	0.9±0.4	0.2±0.4
Average total filamentous fungal colony (CFU/L)	17750±13081.5	52.5±31.8	7.8±1.1	3.3±1.1
Bacterial community				
Total number of OTUs	6152	3736	2207	995
Average number of OTUs per sample	1744.5±482.6	968.3±364.9	546.4±238.1	192.1±68.7
Average bacterial abundance (log genome equivalent/mL)	5.5±0.4	3.4±0.4	2.9±0.3	1.2±0.3
Average heterotrophic plate count (CFU/mL)	501.7±120.2	0.5±0.7	ND	ND
pH	7.5±0.4	7.5±0.5	7.6±0.4	7.6±0.2
Conductivity (µS/cm)	279.7±79.7	304.5±97.8	311.9±66.0	303.1±75.8
Free chlorine residual (mg/L)	ND	ND	ND	0.5±0.3

ND: not detected

*Post-sedimentation samples represent water after 24 hours sedimentation

In summary, our results demonstrated that disinfection shaped both the final bacterial alpha- and beta-diversity, while the final fungal alpha- and beta-diversity was shaped by the media filtration. Previous research identified a similar trend with the bacterial community structure changing in response to addition of secondary disinfection in a premise plumbing system [49], but no change in fungal community structure of the same study site was found after starting secondary disinfection [50].

3.3.4 Fungal Taxonomy

Fungal ITS1 sequences were classified to 1043 fungal genera across all samples. Three phyla were detected with 100% positivity: Ascomycota, Basidiomycota, and Zygomycota. These three phyla represented an average relative abundance of 98.4±1.4%, dominating the fungal community. Fungal taxonomy results for these phyla are shown in Figure 3.4.

The average relative abundance of the Ascomycota phylum was 66.2±19.3% in raw water, 55.9±16.8% post-sedimentation, 56.5±19.8% post-filtration, and 70.7±23.9% post-disinfection, with no statistically significant change through the water treatment process (p-values > 0.05). Within the Ascomycota phylum, the most abundant genera were *Penicillium*, *Aspergillus*, *Alatospora*, *Taphrina*, and *Tuber*, with 100% positivity. *Penicillium* spp. represented an average relative abundance of 1.5±1.8% in raw water, 9.3±13.5% post-sedimentation, 25.2±32.5% post-filtration, and 41.3±38.3% post-disinfection, with a significant increasing trend comparing raw water to post-disinfection water ($p < 0.05$) (Figure 3.4). The average relative abundance of *Aspergillus* spp. was 0.6±0.8% in raw water, 1.2±1.1% post-sedimentation, 1.2±1.2% post-filtration, and 3.6±2.1% post-disinfection. Similar to *Penicillium* spp., the relative abundance of *Aspergillus* spp. significantly increased from raw water to post-

disinfection water ($p < 0.05$); including a significant increase in relative abundance was observed after disinfection ($p < 0.05$) (Figure 3.4). *Alatospora* spp. and *Taphrina* spp., with overall average relative abundance of 2.1±3.9% and 2.8±5.8% respectively, did not show significant change in relative abundance through the treatment processes (p-values > 0.05). A decreasing trend of *Tuber* spp. relative abundance was observed from 19.0±24.5% in raw water to 8.0±7.0% post-disinfection, yet without statistical significance (p-values > 0.05).

The average relative abundance of the Basidiomycota phylum was 23.1±19.2% in raw water, 33.9±19.1% post-sedimentation, 35.7±20.1% post-filtration, and 22.0±17.1% post-disinfection, with no significant change through the treatment process (p-values > 0.05). *Mrakia* and *Peniophora* were the two genera within the Basidiomycota phylum detected with 100% positivity and no significant change of relative abundance across the water treatment process was observed for these two genera (p-values > 0.05), mirroring the trend on phylum level (Figure 3.4). *Peniophora* spp., a soil fungus belongs to Basidiomycota [51], was previously detected in tap water samples [50, 52].

The average relative abundance of the Zygomycota phylum was 8.4±7.1% in raw water, 8.6±8.7% post-sedimentation, 6.7±10.5% post-filtration, and 6.1±7.8% post-disinfection. No statistically significant change was observed through the treatment process (p-values > 0.05). The *Schizangiella* and *Basidiobolus* genera belonging to Zygomycota were detected with 100% positivity, and relative abundance significantly decreased through the treatment processes (p-values < 0.05).

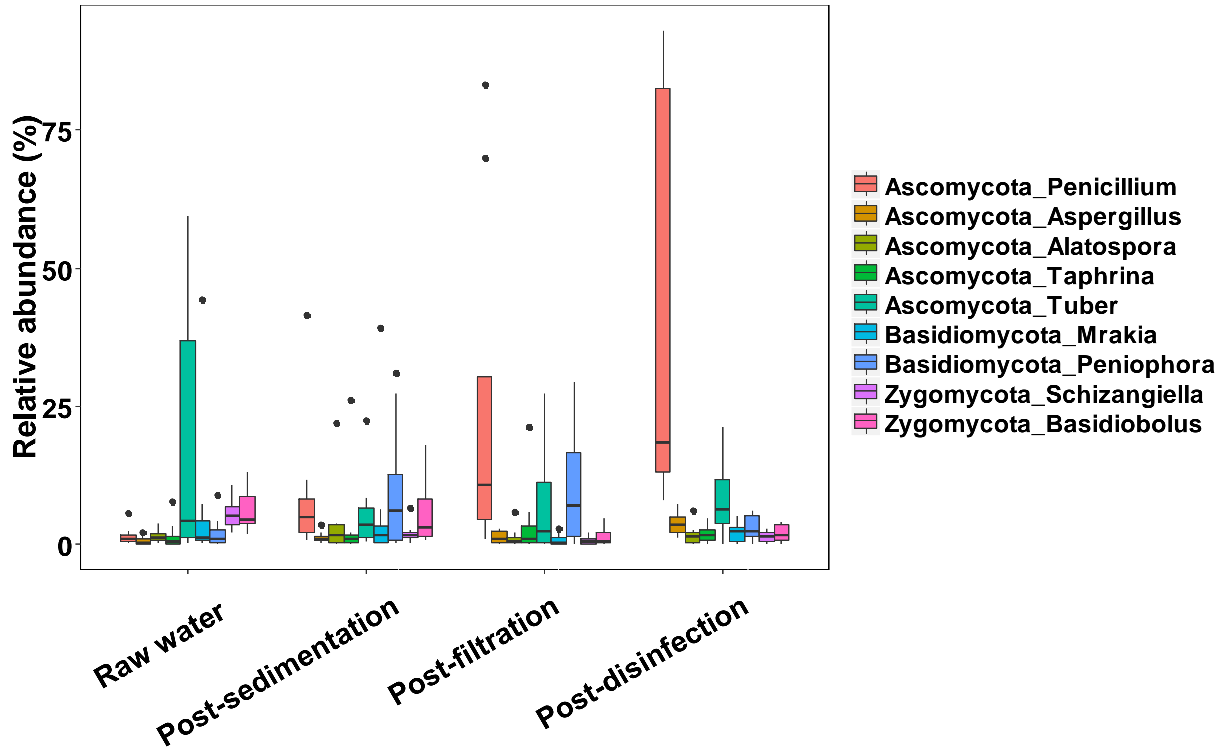

Figure 3.4 Boxplot of core fungal taxa relative abundance through the water treatment processes train; post-sedimentation samples represent water after 24 hours sedimentation

Isolation of the fungal phyla Ascomycota, Basidiomycota, and Zygomycota from untreated surface water has previously been reported [53-55]. However, the effect of centralized water treatment process on the fungal community structure in drinking water had not been previously evaluated. *Penicillium* spp. and *Aspergillus* spp. have been widely isolated from drinking water [6, 13, 56, 57]. Our results demonstrated that the relative abundance of *Penicillium* spp. and *Aspergillus* spp. significantly increased through the water treatment process, especially post-disinfection, suggesting that these two genera were less efficiently removed by the conventional water treatment process, or more persistent to the selection pressure posed by water treatment processes. Previous studies have suggested *Penicillium* and *Aspergillus* produce

unicellular spores with relatively small size (1.8-6 μm × 2-4.5 μm as L × W) [58-60]. The small spore size may facilitate the passage of *Penicillium* and *Aspergillus* through sedimentation and media filtration while other fungal groups with larger spore sizes were removed. Further research are needed to investigate the role of fungal cell size in the fungal community structure change along the drinking water treatment process. Different fungal species demonstrate varying resistance to chlorine disinfection [61]. Chlorine inactivation of species belonging to *Aspergillus*, *Penicillium*, *Cladosporium*, and *Phoma* genera in water were previously compared and the evaluated species of *Aspergillus* and *Penicillium* demonstrated higher chlorine resistance than the other genera [62]. It has been suggested that *Penicillium* and *Aspergillus*, which belong to the family Trichocomaceae, might be more resistant to chlorine inactivation and less effectively removed during disinfection; however, only a limited number of fungal species had been compared for resistance to water disinfection [61, 62]. Further investigations of disinfection of different waterborne fungal species are still needed to inform better control of fungi in drinking water.

In addition to fungi previously abundantly found in water systems, soil-associated fungal taxa were likely introduced to raw water through soil sediments and plant debris, and remained through the treatment process. Specifically, *Alatospora* spp. was previously isolated from decaying leaves and described as an aquatic hyphomycetes genus [63]; *Taphrina* spp., *Tuber* spp., *Mrakia* spp., and *Basidiobolus* spp., which were previously found in soil [64-69], were detected in the present study as core taxa.

3.3.5 Bacterial Taxonomy

Bacterial taxonomy was included in this evaluation to enable comparison of observed trends to previous results. 16S rRNA sequences were classified to 58 bacterial phyla and three archaeal phyla. Five bacterial phyla were detected in all samples (100% positivity): Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria, and Firmicutes. The Nitrospirae phylum was detected in all samples except one post filtration sample, also representing 100% post-disinfection positivity. These six phyla represented an average relative abundance of 97.0±2.7%, dominating the bacterial community. The abundance of core taxa throughout the treatment process is shown in Figure 3.5.

Proteobacteria, which had been widely detected in drinking water as a core taxon [3, 10, 70], dominated the bacterial community in all samples, representing an average relative abundance of 57.6±11.4% in raw water, 55.1±10.3% post sedimentation, 66.2±19.9% post filtration, 63.3±19.4% post disinfection, with no statistically significant change in relative abundance through the treatment process (p-values > 0.05). Within Proteobacteria, Alpha-, Beta-, and Gammaproteobacteria were the most abundant classes and were detected with 100% positivity. The average relative abundance of Alphaproteobacteria was 9.5±2.7% in raw water, 10.4±7.1% post sedimentation, 30.5±30.1% post filtration, and 25.6±25.4% post disinfection. Alphaproteobacteria was previously found to be a dominant bacterial class within filter media, suggesting media filtration may provide potential survival niche for Alphaproteobacteria, or conversely that Alphaproteobacteria are less efficiently removed by the filtration process [10, 12]. In this study *Sphingomonas* spp. was the most abundant genus affiliated to Alphaproteobacteria. Previous research demonstrated the wide occurrence of Sphingomonas spp. in drinking water system biofilms and its important role in biofilm formation [71-73], suggesting

that *Sphingomonas* spp. had potential in filtration media biofilm formation and subsequently influenced the post filtration bacterial community structure. Betaproteobacteria had an average relative abundance of 41.1±10.1% in raw water, 35.5±11.9% post sedimentation, 31.3±15.8% post filtration, and 19.6±15.0% post disinfection, with a significant decreasing trend comparing raw water to post disinfection water ($p < 0.05$) (Figure 3.5). The average relative abundance of Gammaproteobacteria was 5.2±2.4% in raw water, 8.5±11.2% post sedimentation, 4.1±4.2% post filtration, and significantly increased to 17.1±11.3% post disinfection ($p < 0.05$), indicating a selection effect favoring Gammaproteobacteria posed by disinfection. *Acinetobacter* spp. was found to be the most abundant Gammaproteobacteria affiliated genus with increased relative abundance post disinfection. Several strains of *Acinetobacter* spp. were previously found to be able to survive without significant inactivation when exposed to 4 mg/L free chlorine up to 2 minutes [74]. Previous study also demonstrated higher resistance by Gammaproteobacteria than Alpha- and Betaproteobacteria to the same level of free chlorine [75]. The differential resistance to free chlorine likely drove the shift in the composition of the Proteobacteria phylum during the disinfection step.

The average relative abundance of the Actinobacteria phylum was 18.5±10.3% in raw water, 12.7±8.6% post sedimentation, 6.7±4.9% post filtration, and 7.1±5.6% post disinfection. The Actinobacteria class was the dominant class and detected with 100% positivity, with an average relative abundance of 17.0±9.4% in raw water, 12.3±8.6% post sedimentation, 6.5±4.8% post filtration, and 6.5±5.4% post disinfection. A significant trend of decreasing relative abundance was observed comparing raw water to post disinfection water (p-values < 0.05) (Figure 3.5). Previous studies also found decreased relative abundance for the Actinobacteria phylum comparing treated drinking water to raw water [10, 11].

The Bacteroidetes phylum represented average relative abundance of 15.4±2.7% in raw water, 24.7±8.9% post sedimentation, 23.8±15.8% post filtration, and 2.0±2.7% post disinfection. A trend of decreasing relative abundance, similar to Actinobacteria, was found comparing raw water to post disinfection water ($p < 0.05$). Additionally, Bacteroidetes had significantly increased relative abundance post sedimentation ($p < 0.05$), and significantly decreased relative abundance post disinfection ($p < 0.05$). Cytophagia and Saprospirae were the most abundant bacterial classes within the Bacteroidetes phylum and reflected similar trend of relative abundance with phylum level.

The Cyanobacteria and Firmicutes phyla shared similar trends that represented low relative abundance without a statistically significant change (p-values > 0.05) before disinfection, but significantly increased after disinfection (p-values < 0.05). The average relative abundance of Cyanobacteria was 1.0±1.3% in raw water, 3.3±4.9% post sedimentation, 0.7±1.1% post filtration, and statistically significantly increased to 10.7±10.4% post disinfection ($p < 0.05$). 4C0d-2 was the most abundant class within Cyanobacteria, and showed a similar trend of relative abundance with phylum level. The Firmicutes phylum represented an average relative abundance of 1.1±0.9% in raw water, 1.0±1.3% post-sedimentation, 0.4±0.7% post-filtration, and statistically significantly increased to 10.8±7.5% post-disinfection ($p < 0.05$). Within the Firmicutes phylum, Bacilli and Clostridia were the most abundant classes and a statistically significant increase in relative abundance was observed for Bacilli and Clostridia following disinfection (p-values < 0.05), mirroring the phylum level trend (Figure 3.5). The high resistance to chlorine of Bacilli and *Clostridium* spp., the most abundant Clostridia affiliated genus in this study, was demonstrated by previous studies [76, 77], and likely favored the survival of these species during the disinfection step.

The Nitrospirae phylum, represented entirely by the Nitrospira class, represented an average relative abundance of 0.1±0.1% in raw water, 0.2±0.1% post sedimentation, 0.5±0.7% post filtration, and 5.1±6.7% post disinfection. Increased average relative abundance post disinfection was found, yet without statistical significance (p-values > 0.05) (Figure 3.5). *Leptospirillum* spp. was identified in all eight post-disinfection samples but were only detected in 10 out of 24 (41.7%) of pre-disinfection samples, suggesting *Leptospirillum* spp. may have had survival niche within the disinfection unit operation. The *Leptospirillum* genus, which contains iron-oxidizing species [78], was previously found in cast iron water pipe biofilm and suggested to contribute to iron pipe corrosion [79]. Further evaluating resistance of *Leptospirillum* spp. to disinfectant might help to explain and inform its presence and control in post-disinfection water.

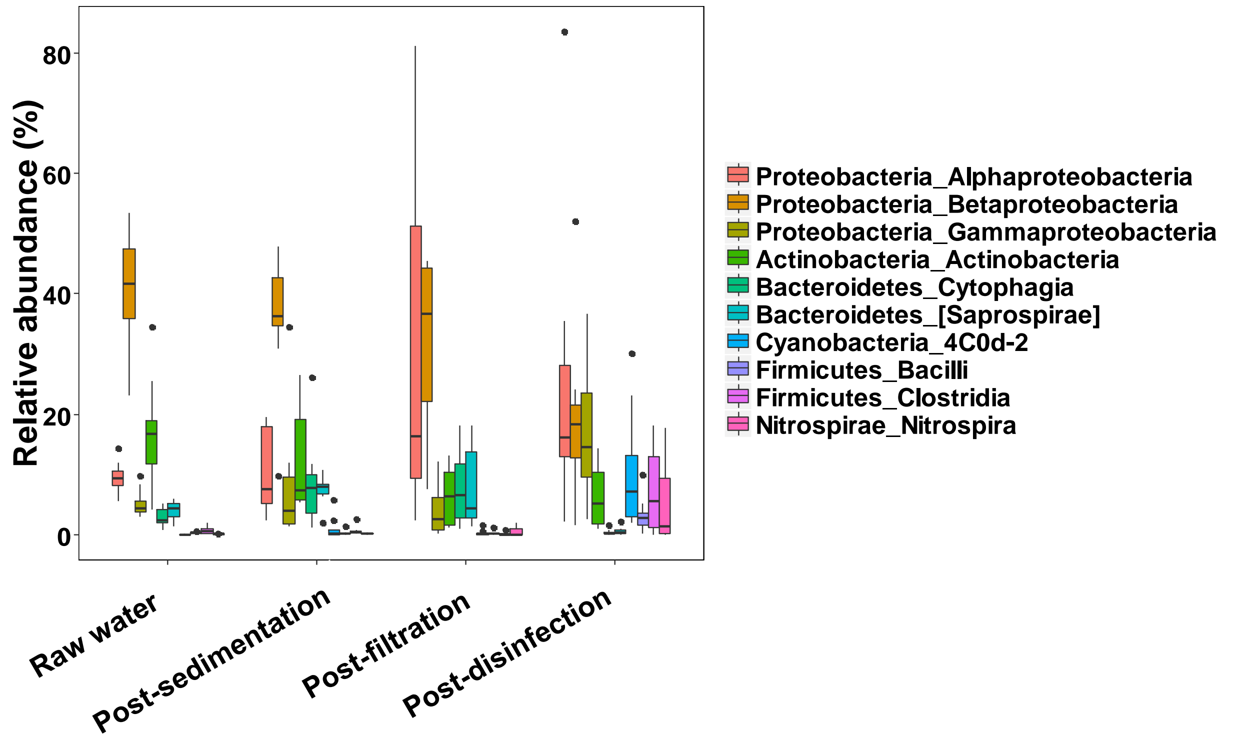

Figure 3.5 Boxplot of core bacterial taxa relative abundance through the water treatment processes train; post-sedimentation samples represent water after 24 hours sedimentation

3.4 SUMMARY AND CONCLUSIONS

In the current study we applied molecular approaches to investigate microbial abundance, diversity, and taxonomy following drinking water treatment plant unit operations. Both bacterial and fungal abundance were observed to decrease following each treatment step, with the exception of fungal abundance following disinfection. A similar trend was observed for alpha- and beta-diversity for both bacteria and fungi, where each treatment operation decreased microbial community richness and altered microbial community beta-diversity, with the exception of disinfection for the fungal community. The bacterial community was dominated by phyla Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria, Firmicutes, and Nitrospirae. The fungal community was dominated by phyla Ascomycota, Basidiomycota, and Zygomycota. *Penicillium* spp. and *Aspergillus* spp., which have been previously isolated from drinking water, were detected in samples through the treatment process and had increasing relative abundance. Ultimately, disinfection by free chlorine shaped the bacterial community entering the distribution system. Unlike bacteria, the fungal community in the treated drinking water was more significantly affected by media filtration. These results demonstrate the role of drinking water unit operations in shaping both the fungal and bacterial communities, indicating the potential of influencing microbial communities in downstream water distribution systems. The results also highlighted the difference in structure variation between bacterial and fungal communities. More studies evaluating impact to microbial community structure by different water treatment

approaches, such as different disinfectants, may further inform the generalization of engineering approaches' impact to drinking water microbiome.

4.0 FUNGAL DIVERSITY IN A HOSPITAL PREMISE PLUMBING SYSTEM TREATED WITH ON-SITE MONOCHLORAMINE

This work has been published as:

Ma X, Baron JL, Vikram A, Stout JE, Bibby K. Fungal Diversity and Presence of Potentially Pathogenic Fungi in a Hospital Hot Water System Treated with On-Site Monochloramine. Water Research, 2015. 71(15): p. 197-206

4.1 INTRODUCTION

Fungi are common but poorly understood inhabitants of drinking water treatment and distribution systems. Reports have previously detailed the occurrence of diverse fungal communities in drinking source water [1], the passage of fungi through conventional water treatment [2], and the isolation of fungi from downstream water distribution systems [3-6]. Potentially pathogenic fungi such as *Aspergillus* spp., *Candida* spp., and *Fusarium* spp. have been identified in hospital water systems [7-10] and drinking water may function as a mode of transmission for fungi [9]. Fungi in healthcare facility premise plumbing systems are of greater concern as these facilities usually have immunocompromised residents, including transplant patients, which are more susceptible to infection from pathogenic fungi [11]. As the number of immunocompromised, susceptible patients increases, large facilities (especially hospitals) have

begun to adopt secondary water disinfection systems in order to control opportunistic pathogens. The effect of these treatments on the fungal ecology of the drinking water system remains unknown. There have been a limited number of studies investigating the fungal ecology of hot water systems [4, 9, 12], and no previous studies investigating the influence of on-site disinfection systems on fungal ecology.

Previous studies of fungal ecology in municipal drinking water have been primarily culture based, limiting throughput and potentially biasing results based upon selection of culture media or isolation approach [13]. Next generation sequencing methods have dramatically expanded researchers' ability to evaluate microbial diversity in environmental samples. Studies sequencing the fungal internal transcribed spacer (ITS) region have been conducted to investigate fungal ecology in soil [14, 15] and aerosols [16, 17]. Additionally, it is desirable to move towards higher-throughput sequencing approaches (e.g. Illumina) for fungal ITS1 sequencing, enabling greater sampling depth, breadth, and economy.

In the current study, we examined fungal diversity in a hospital hot water system, located in Pittsburgh, PA, that initiated on-site monochloramine treatment as secondary disinfection to control *Legionella* spp. colonization. The primary goal of this study was to investigate the fungal diversity within the hospital premise plumbing hot water system and the response of the fungal community to monochloramine treatment. Additionally, the accuracy of fungal identification by Illumina sequencing of the ITS1 gene region was evaluated by sequencing the ITS1 region of five fungal pure cultures.

4.2 MATERIALS AND METHODS

4.2.1 Sample collection and DNA extraction

The study site was a 12-floor, 495-bed tertiary care hospital complex that receives chlorinated municipal cold water. The hot water system was treated with monochloramine injected by an on-site monochloramine generation system (Sanipur, Lombardo, Flero, Italy). Monochloramine concentration (mg/L as Cl_2) was measured during the sampling with a Hach DR/890 colorimeter, and detailed description of the system and measurement of other physical parameters were described in earlier study [18, 19]. The monochloramine injection was initiated on September, 2011. Baseline water samples were taken three months prior to and immediately prior to the introduction of on-site monochloramine injection, and samples were also taken monthly for the first 6 months of treatment. These monthly samples were collected in June (baseline), September (baseline), October, November, and December in 2011, January, February, and March in 2012. Each month, 27 hot water outlets throughout the hospital were used for hot water sample collection (4 standard faucets on Floors 3-5; 7 sensor faucets on Floors 6-7; 7 standard faucets and showers on Floors 6-7, 6 standard faucets on Floors 8-12, and 2 hot water tank outlets and the hot water return line outlet). Before DNA extraction, all water samples were grouped into 5 pools for each month: Pool 1 (standard faucets from Floors 3-5), Pool 2 (sensor faucets from Floors 6-7), Pool 3 (standard faucets/shower from Floors 6-7), Pool 4 (standard faucets from Floors 8-12), and Pool 5 (hot water tanks and hot water return line). The sampling strategy is summarized in Table 4.1. In total, 40 pooled samples were generated in this manner for the 8 months of sampling. Genomic DNA was then extracted from each of the pooled samples. This study builds upon environmental samples from a previous investigation which focused on

monochloramine's effect bacterial ecology; a detailed description of sampling, water quality testing and DNA extraction procedures can be found in the previous study [18]. Due to the nature of the sampling method, absolute quantification was not possible.

In addition to environmental sampling, DNA was analyzed from five pure cultures of fungi with known identities: *Alternaria alternata* (PEM 01043), *Aspergillus fumigatus* (ATCC 34506), *Cladosporium cladosporioides* (ATCC 16022), *Epicoccum nigrum* (TU BL-3), *Penicillium chrysogenum* (CAES PC-1), to test the accuracy of the sequencing method of fungal identification described in this study. Pure culture fungal DNA was obtained from the Lab of Dr. Jordan Peccia (Yale University, CT).

4.2.2 PCR and Illumina sequencing

The ITS1 region of the extracted DNA was amplified using forward primer ITS1FI2 and reverse primer ITS2 [14, 20]. A previously described combinatorial primer labeling approach [21] was used to label samples for multiplex sequencing on the Illumina MiSeq platform. The complete sequence of forward and reverse primers are summarized in Appendix B Table B1.

PCR reactions were performed in 20 μL reaction mixtures containing 1 μL of sample DNA, 10 μL of 2x DreamTaq Master Mix polymerase (Thermo Scientific), and 0.25 μM of each of the combinatorial ITS1FI2 and ITS2 primers. Temperature condition was 15 minutes denaturation at 95°C, followed by 40 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds extension at 72°C, and a final elongation at 72°C for 5 minutes. PCR reaction for each sample includes three replicates, and replicates of each reaction were pooled after PCR amplification and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). Negative controls in which the template DNA was replaced with 1 uL sterile

molecular biology grade water were included in each PCR run to ensure reagents and equipments were not contaminated. Gel visualization of AMPure XP beads purified PCR products were performed for quality inspection.

The purified PCR products of environmental samples were quantified by Qubit 2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Invitrogen), and then normalized and pooled based on equal molarity. One additional purification was performed for the final pool of PCR products from environmental samples with Agencourt AMPure XP magnetic beads. Then the purified library was loaded to Illumina MiSeq Desktop Sequencer (Illumina) for sequencing by using customized sequencing primers. Sequences of the sequencing primers were summarized in Appendix B.

For PCR products of pure culture fungal DNA, the purified products were purified by gel extraction again, then normalized and pooled based on equal molarity to form the final pure culture sequencing library and loaded to Illumina MiSeq Desktop Sequencer using the same customized sequencing primers described above in another sequencing run.

4.2.3 Bioinformatics

Raw ITS1 sequence data were demultiplexed and trimmed to remove primer sequences and sequences below quality score of 20 utilizing QIIME 1.7.0 [22]. The trimmed sequences were compared to a named fungal ITS sequences database [23] using BLASTn ver. 2.2.19 [24], the database was manually amended to remove teleomorphs and suspected misannotated sequences, and trimmed sequences are listed in Appendix B Table B1. BLASTn outputs were then subjected to fungal taxonomic identification tool FHiTINGS [25] to sum and sort results based on

taxonomic ranks from kingdom to species. Sequences are available under MG-RAST [26] accession number 4583532.3.

Persistence across all samples (= $N_{positive}/N_{total}$; $N_{positive}$: number of positive samples, N_{total}: total number of samples) was computed for each assigned genus. A Persistence-Abundance plot was drawn by plotting the persistence against the maximum relative abundance of the genus in any one sample. We utilized a previously suggested threshold of ≥50% persistence [27-30] to define the core fungal genera in this hospital hot water system. The accuracy of pure culture sample annotation, true identification ratio (TIR) which is the fraction of sequences assigned to correct taxonomy, was calculated based on pure culture fungal ITS1 DNA sequencing outputs (TIR = N_{true}/N_{total}, N_{true}: number of sequences assigned to the correct taxonomy, N_{total}: total number of sequences) [31]. To assess the alpha-diversity of the fungal community, the Shannon's equitability index was calculated based on the assigned genera and associated relative abundance [32]. One-way ANOVA was used to compare genera number before and after monochloramine treatment. To assess the beta-diversity of the environmental fungal community, Bray-Curtis dissimilarity based on taxonomy identities and relative abundances was computed using the R package Ecodist, and principal coordinate analysis based on Bray-Curtis dissimilarity was performed [33, 34].

4.3 RESULTS

4.3.1 PCR and sequencing results of environmental samples

PCR amplifications were performed for each sample pool. Pool 1 through Pool 4 samples were positive for PCR amplification, except Pool 2 December 2011 and Pool 3 March 2012. Pool 5 (hot water tank samples) was negative for all PCR reactions, potentially due to PCR inhibitors, such as calcium from hot water tank calcium residual [35], or higher temperature within the hot water tank inhibiting fungal growth [4]. No-template, negative controls were used in the PCR to ensure reagents and equipments were not contaminated and all negative controls were negative for amplification. In total, 119,300 sequence reads were used for taxonomic classification after quality trimming (Table 4.1).

Table 4.1 Sequencing results after quality trimming

Sampling pool	Sample Description	Number of samples	Total Number of Reads	Average Read Per Sample	Median Read Length
Pool 1	Standard faucets (Floors 3-5)	8	12378	1547	251
Pool 2	Sensor faucets (Floors 6-7)	7	25257	3608	251
Pool 3	Standard faucets and Showers (Floors 6-7)	7	41535	5934	251
Pool 4	Standard faucets (Floors 8-12)	8	16314	2039	251

4.3.2 Sequencing accuracy

Pure culture fungal DNA was sequenced to evaluate the accuracy of taxonomy assignments. The method used in this study had high true identification ratios at the genus rank; however, the true identification ratios at the species rank were lower (Table 4.2, details of error rate calculation in Appendix B Table B2 & 3). The high true identification ratio on genus rank indicate the fungal taxonomy identification results based on ITS1 region is reliable to the genus level, consistent with previous work on the annotation accuracy of ITS1 sequences [31].

Table 4.2 True identification ratio (TIR) of the present ITS1 sequencing method on genus level and species level

Tested Fungi	Genus rank TIR (%)	Species rank TIR (%)
Alternaria alternata	96.91	1.40
Aspergillus fumigatus	99.76	0.79
Cladosporium cladosporioides	95.76	0.68
Epicoccum nigrum	99.77	99.77
Penicillium chrysogenum	99.61	0.39

4.3.3 Fungal community taxonomic assignment and core genera

Taxonomic assignment results indicated that *Penicillium* was the dominant fungal genus, with an average relative abundance of 88.89% (± 6.37%) (Figure 4.1, Table 4.3). The fungal community in the building hot water system was highly diverse, with 202 different fungal genera being identified (Appendix B Table B4). The core fungal genera were identified based upon the percentage of positive identifications (persistence). We adopted a previously suggested 50% persistence threshold to identify core and occasional genera within the fungal community [27]. The core fungal biome, as indicated by persistence, consisted of the genera *Penicillium* (100%), *Aspergillus* (90%), *Peniophora* (56.67%), *Cladosporium* (50%), and *Rhodosporidium* (50%) (Figure 4.2). Two genera were found to have persistence close to but lower than 50%: *Aureobasidium* (43.33%), and *Fusarium* (40%) (Figure 4.2).

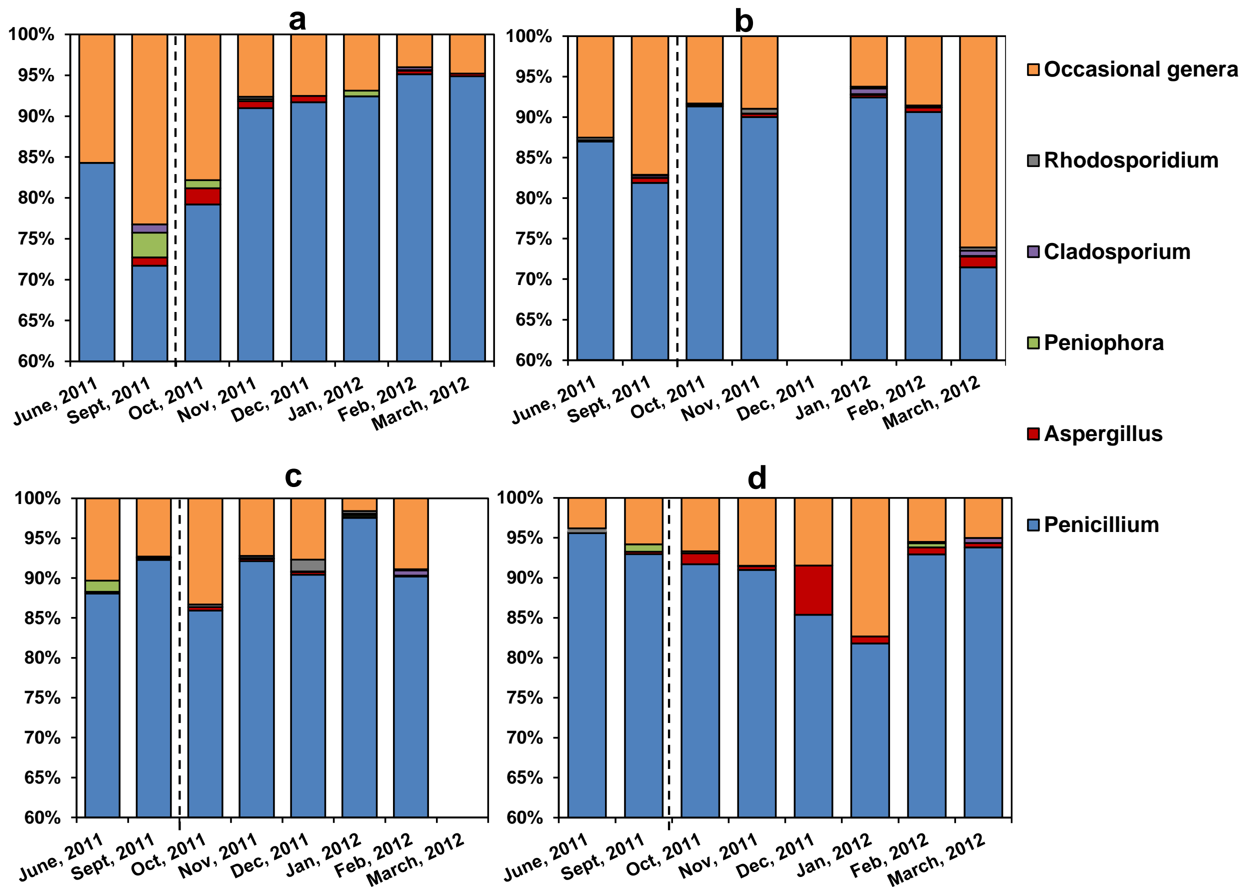

Figure 4.1 Relative abundance of fungal genera within sample Pools 1 through 4: Pool 1-Standard faucets on Floor 3-5 (Panel a); Pool 2-Sensor faucets on Floor 6-7 (Panel b); Pool 3-Standard faucets and showers on Floor 6-7 (Panel c); Pool 4-Standard faucets on Floor 8-12 (Panel d). Columns on the left side of dashed lines represent samples collected before on-site monochloramine treatment; Pool 2 December and Pool 3 March samples are excluded due to negative PCR amplification.

The majority of genera detected were with low persistence (< 40%) and low relative abundance (0.003% - 3.80%). The sole exception was the genus *Pichia*, whose maximum relative abundance was 19.78% (Pool 2, March 2012). However, the average relative abundance of *Pichia* spp. was 0.87% with a 30% persistence; thus *Pichia* was classified as an occasional genus.

Table 4.3 Core fungal genera and genera with persistence between 40-50% detected in this work with previous isolation report and/or potential pathogenicity

Genus Sequences Detected in This Work	Average Relative Abundance (± Standard Deviation)	Previously Reported Isolation From Water System	Previously Reported Pathogenicity
Penicillium	88.89±6.37%	Isolated from municipal water distribution system biofilm and treated drinking water [4, 6, 7, 36-38]	Some species are reported pathogenic [39, 40]
Aspergillus	0.71±1.13%	Isolated from biofilm within municipal water distribution system and treated drinking water; presence in hospital water system is also reported and suggested as a potential source of nosocomial Aspergillus infection [4, 6, 7, 36, 38, 41]	Some Aspergillus species cause invasive aspergillosis [7, 39, 42, 43]
Peniophora	0.29±0.62%	18S gene sequences of *Peniophora* was previously detected in tap water [12]	N/A
Cladosporium	0.16±0.28%	Isolated from biofilm within municipal water distribution system and treated drinking water [4, 6, 36, 38]	Reported infection case [44]
Rhodosporidium	0.18±0.30%	Isolated from raw source water [45]	*Rhodosporidium toruloides* was suggested as opportunistic pathogen [46]
Aureobasidium	0.25±0.90%	Isolated from biofilm within municipal water distribution system and treated drinking water [4, 6, 38]	*Aureobasidium pullulans* is reported to cause phaeo-hyphomycosis [39]
Fusarium	0.14±0.34%	Isolated from treated drinking water, including hospital water system [4, 7, 37, 38, 47]	Contains opportunistic pathogens [39]

N/A: Not available

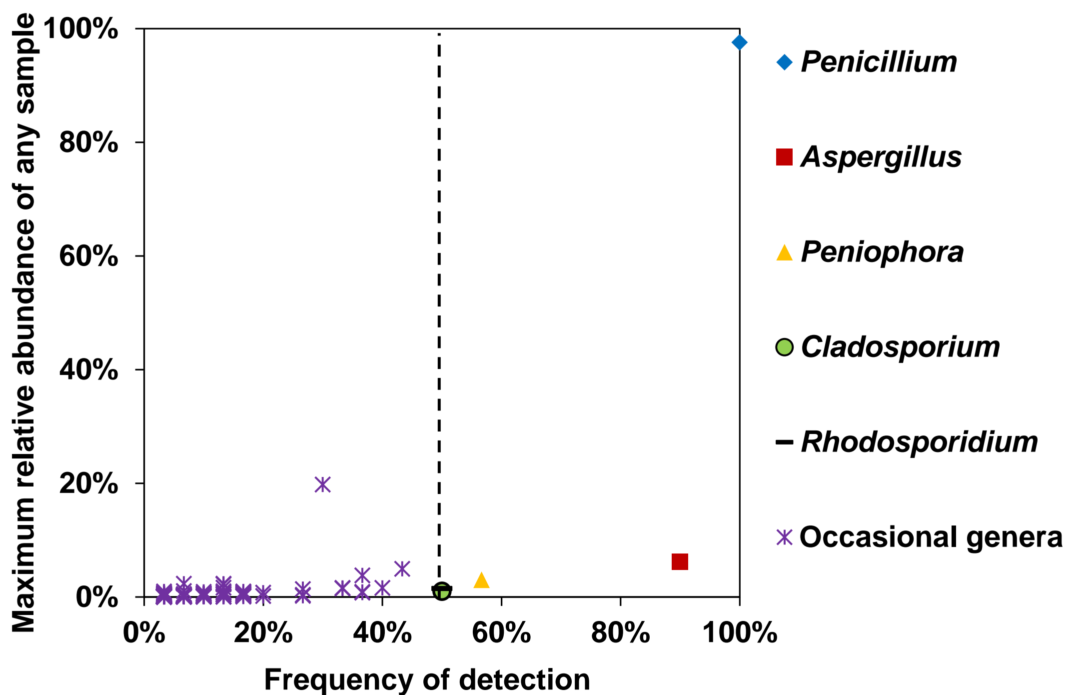

Figure 4.2 Persistence-Abundance plot of fungal genera detected. The dashed line represents the 50% cut off point used to determine core fungal genera; symbols to the right of this line represent the core fungal genera found in our samples

4.3.4 Alpha- and beta-diversity of the fungal biome

The fungal community structure was highly uneven due to the consistent high abundance of *Penicillium*. The equitability index values for all samples ranged from 0.06 to 0.46 with an average of 0.19 ± 0.10 (Appendix B Table B5), where a value of 1 would indicate an ideally even community with all members in equal abundance [32]. The number of genera identified before and after monochloramine addition did not change significantly (p-value = 0.795) (Figure

4.3). As the genus *Aspergillus* contains many pathogenic species, which can cause nosocomial infections and be transmitted through water [7, 43], we investigated the relative abundance of *Aspergillus*. We found no statistically significant difference in the relative abundance of *Aspergillus* before and after monochloramine treatment (p-value = 0.258) (Figure 4.4).

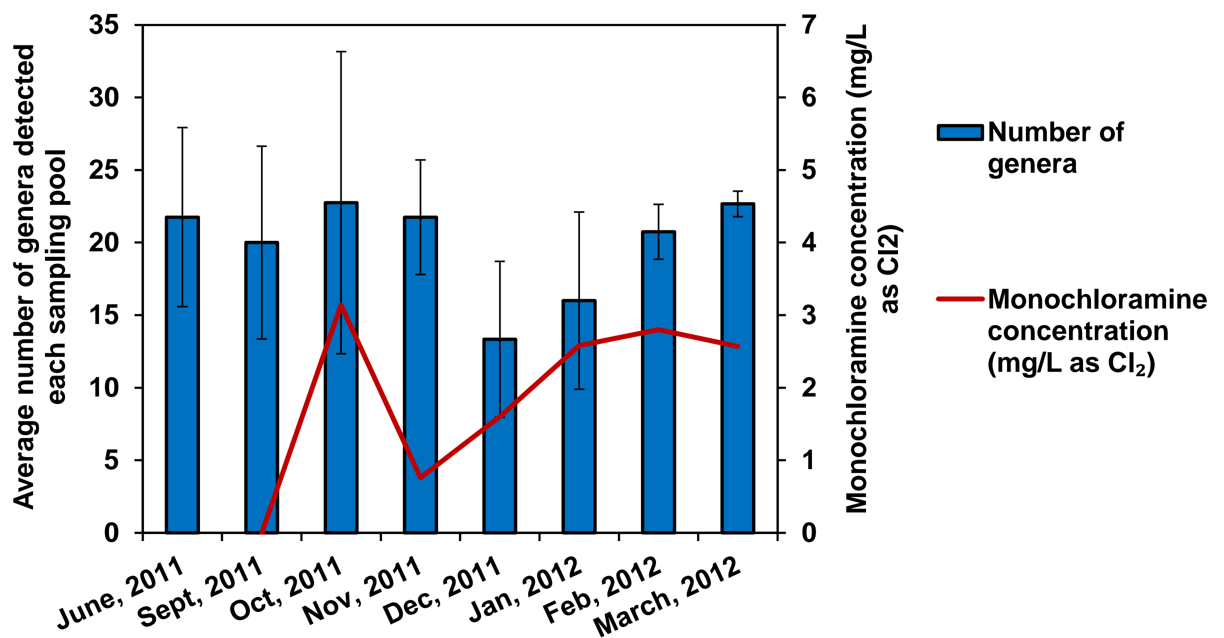

Figure 4.3 Average number of genera detected (blue columns) and monochloramine concentration in hot water as mg/L of Cl2 (red line); monochloramine addition began September 26th 2011 (Appendix B Table B6)

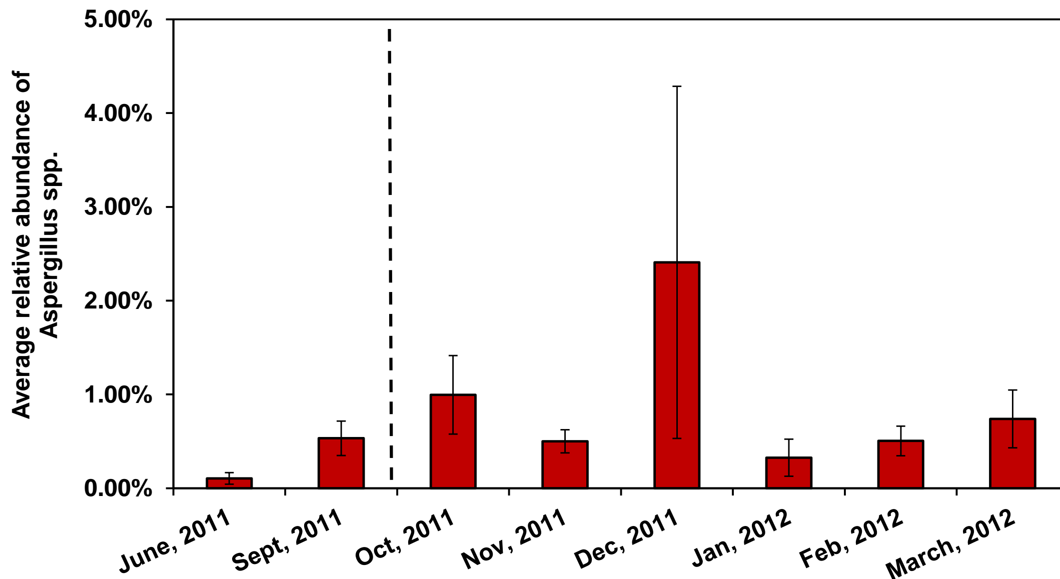

Figure 4.4 Average relative abundance of *Aspergillus* spp. Columns on the left side of dashed lines represent baseline samples collected before on-site monochloramine treatment

Bray-Curtis dissimilarity was calculated based on genus taxonomy assignments and associated relative abundance data of each sample. Principal coordinate analysis based on Bray-Curtis dissimilarity was used to visualize sample beta-diversity (Figure 4.5). Samples taken before monochloramine treatment cluster together with samples taken after monochloramine treatment, and further ADONIS analysis (permutational multivariate analysis of variance) [48] indicated there was no significant difference between fungal community structure before and after monochloramine treatment ($p > 0.05$). This result suggests that monochloramine addition did not impact fungal community structure in the hospital hot water system.

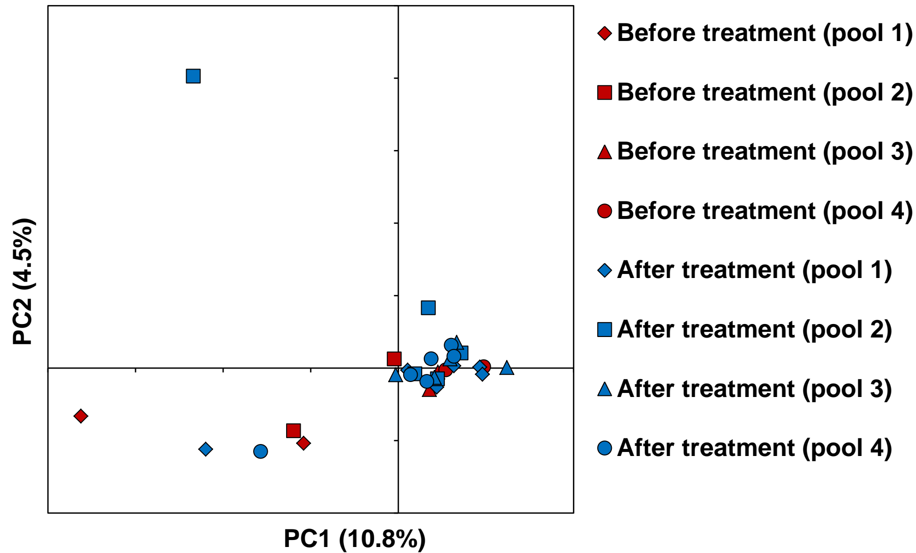

Figure 4.5 Principal Coordinate Analysis based on Bray-Curtis dissimilarity. Outliers represent samples with a larger percentage of occasional genera.

4.4 DISCUSSION

The number of fungal genera identified in the present work was higher than previous culture-based drinking water fungal ecology studies [4, 6]. This may be due to the large fraction of waterborne fungi that are not culturable [49]. The study hospital's hot water system was supplied by municipally treated surface river water, where a large and diverse fungal community can be found [2, 4]. Non-viable fungal DNA fragments may also be analyzed using a sequencing based approach, inflating the number of fungal genera identified. Thus, an approach based on

persistence was utilized to identify fungi that are likely colonizing the premise plumbing hot water system.

4.4.1 Core hot water fungal genera and potential pathogenicity

As the fungal community was highly dominated by *Penicillium* spp. and other genera only represented a small fraction of the whole community, we chose to identify core fungal genera relying on persistence rather than abundance. We chose a persistence threshold of ≥50% based upon the data distribution (Figure 4.2) [27-30].

Penicillium spp. and *Aspergillus* spp. have been isolated from drinking water by many studies [4, 6, 7, 36, 38, 41]. A previous culture-based isolation study suggested that the fungal biome in a Norwegian drinking water supply system was dominated by *Penicillium* spp., while *Aspergillus* spp. was also frequently isolated [4]. *Trichoderma* spp. was also reported as a major fungal genus in the Norwegian drinking water study, as it was frequently isolated from cold tap water but was less frequently detected in hot tap water [4]. In the present study *Trichoderma* spp. were also detected but with low persistence and low relative abundance (3.33% persistence, classified as occasional genus), consistent with the analysis of hot water in the Norwegian study [4]. Another study investigated the fungal biofilms in a municipal (cold) water distribution system in Missouri; it found *Penicillium* and *Aspergillus* to be the most common fungal genera in terms of both abundance and persistence [6]. *Penicillium* spp. and *Aspergillus* spp. were also found to be the two most common fungi recovered in a prospective study targeting pathogenic fungi in hospital water based on culture isolation [9]. In our study, *Penicillium* was found to be the dominate fungal genus present in hot water samples and *Penicillium* spp. and *Aspergillus*

spp. were the two most frequently detected fungi in the hot water supply system (Figure 4.1 & 4.2).

In addition to *Penicillium* spp. and *Aspergillus* spp., other genera within the core fungal microbiome have previously been isolated from drinking water. Although not previously cultured, 18S rRNA sequences belonging to *Peniophora* spp., a plant pathogen, were identified in tap water [12]. *Cladosporium* spp. has been isolated from drinking water and a municipal water distribution system biofilm [4, 6, 9]. Isolation of *Rhodosporidium* in untreated drinking water sources has been previously reported [45]. The two fungal genera (*Aureobasidium* and *Fusarium*) with persistence between 40% and 50% have been isolated from treated drinking water, including taps and showers in a hospital as well as a biofilm within drinking water distribution networks [4, 6, 36, 38, 47].

Many species within the genus *Aspergillus* are known opportunistic human pathogens causing aspergillosis [7, 50-53], and previous long term studies have suggested that water should be considered a potential source of nosocomial aspergillosis [7, 9]. Some *Rhodosporidium* species, such as *R. toruloides*, may cause invasive infection [46]. Most of the core fungal genera we found in this study have been detected in water systems previously and may contain potentially pathogenic species (Table 4.3). Residents of the hospital, including an immunocompromised persons, may be exposed directly to potentially pathogenic fungi when using water, or may receive respiratory exposure via fungal spores and hyphal fragment aerosolization through water taps and showers [4].

Several fungal genera such as *Cladosporium*, *Aureobasidium*, *Fusarium*, *Alternaria*, *Acremonium*, *Candida*, *Cryptococcus* and *Paecilomyces* contain reported pathogenic species [11, 39, 43, 44, 47, 54]. These genera were detected among the occasional group (<50% persistence

across all samples), and their isolation was previously reported from drinking water [4, 6, 9, 36-38, 41, 47]. Although the occasional fungal genera had relatively low persistence across samples and low relative abundance, the potential for pathogenic fungal exposure to immunocompromised patients may still exist.

The studied hot water system receives cold municipal drinking water from a drinking water treatment plant treating surface water. Surface water contains many diverse microorganisms, including fungi. Many fungi may survive the municipal water treatment processes and reach distribution network distal sites [4]. We believe that the core fungal genera, especially *Penicillium* and *Aspergillus*, likely colonize the premise plumbing system as members of biofilms on inner pipe surfaces [4, 6]. As the core fungal genera has an increased likelihood of growing in the hospital's premise plumbing system, patients (especially immunocompromised or those undergoing immunosuppression treatment) have a higher likelihood of exposure to these potentially pathogenic fungi through contact with water. These results demonstrate that hospital hot water system could potentially be a source of diverse opportunistic fungal pathogens.

4.4.2 On-site monochloramine addition did not alter fungal community structure

Fungal community alpha- and beta- diversity remained constant following monochloramine addition (Figures 4.3 & 4.4). A study at the same sampling site focusing on bacterial ecology showed a high level of bacterial community response to monochloramine [18]. Dominant bacterial phyla shifted from Betaproteobacteria to Firmicutes, Alphaproteobacteria, Gammaproteobacteria, Cyanobacteria, and Actinobacteria during on-site monochloramine addition [18]. Contrary to the results for bacterial community structure, the fungal community in this hospital hot water system did not show a significant response to monochloramine, higher

disinfection resistance of core fungal genera observed in the system likely facilitated the lack of significant fungal community structure change. Chapter 5 of the dissertation shows that commonly found waterborne fungi such as *Aspergillus* and *Penicillium* are resistant to monochloramine disinfection, with 3-log_{10} inactivation Ct values (product of monochloramine residual concentration, C and contact time, t) ranging from 90.33 mg•min/L to 531.3 mg•min/L, which are similar to disinfection resistant bacteria *Legionella pneumophila* and *Mycobacterium* spp. [55-60]

As the studied hospital premise plumbing hot water system is a complex engineered system, other engineering factors may also be shaping the fungal microbiome such as pipe material, water age, water chemistry, and water temperature. A previous study based on 18S rRNA gene T-RFLP analysis suggested that disinfectant type, pipe material, and water age interact with each other to shape the water microbiome, and the effect of disinfectant type on eukaryotic microbiome was not apparent [61]. The present study further demonstrates that application of monochloramine as secondary disinfection did not alter the fungal community structure. Future studies using deep sequencing targeting the ITS region would bring additional insights to the effect of engineering controls on the fungal microbiome. Additionally, further controlled studies focusing on fungal ecology in water distribution systems are necessary to understand the factors driving fungal ecology in water systems, and to ultimately engineer a control strategy for opportunistic fungal pathogens.

4.5 SUMMARY AND CONCLUSIONS

Multiple fungal species, including potential opportunistic pathogens, have previously been identified in drinking water based on culture isolation [4, 6, 9]. However, previous studies have been challenged by the limited throughput with which the fungal ecology was examined and potential bias by culture isolation [13]. In the present study, the fungal ecology of a hospital hot water system water was analyzed prior to and following the application of secondary monochloramine disinfection utilizing Illumina sequencing of the fungal ITS1 region. This study represents the first evaluation of the fungal ecology of the hospital hot water system in response to application of secondary water disinfection. The fungal community was dominated by *Penicillium* spp. with an average relative abundance of 88.89% (±6.37%). The core fungal biome contained the genera *Penicillium*, *Aspergillus*, *Peniophora*, *Cladosporium* and *Rhodosporidium*. Many of these genera detected contain known opportunistic pathogens and can pose a risk to hospitalized patients. As measured by alpha- and beta-diversity, the fungal community structure remained constant prior to and following on-site secondary water disinfection using monochloramine. An increasing number of facilities, containing immunocompromised and susceptible populations, are moving towards on-site secondary water disinfection systems to control opportunistic pathogens, such as *Legionella* species. This necessitates the consideration of disinfectant efficacy on a broad suite of opportunistic pathogens to verify the effectiveness of these systems. Our study demonstrates that waterborne fungi are present in many hot water samples, and remain despite monochloramine disinfection with potential implications for opportunistic pathogen management and transmission.

5.0 DISINFECTION KINETICS OF *ASPERGILLUS* AND *PENICILLIUM* BY FREE CHLORINE AND MONOCHLORAMINE IN DRINKING WATER

This work is being prepared for publication as:

Ma, X., Bibby, K., Free Chlorine and Monochloramine Inactivation Kinetics of *Aspergillus* and *Penicillium* in Drinking Water

5.1 INTRODUCTION

Fungi are widely occurring microorganisms in drinking water [1-4]. Chapter 2 of the dissertation reviewed previous studies and demonstrated *Penicillium* spp. and *Aspergillus* spp. are the two most commonly culture isolated fungal genera from drinking water [1-19]. Potential opportunistic pathogens belonging to *Aspergillus* spp., such as *A. fumigatus*, are also frequently isolated from drinking water; and drinking water has been suggested as a potential environmental source of *Aspergillus* infections [2, 6-8, 10, 11, 17, 18]. By using amplified fragment length polymorphism (AFLP), previous study has demonstrated the genetic similarity between *A. fumigatus* clinical isolates and *A. fumigatus* strains isolated from hospital tap water, suggesting the possibility that the *A. fumigatus* was transmitted through tap water [20]. *Aspergillus* spp. is also the second most common cause of nosocomial opportunistic fungal infections, with *A. fumigatus* as the major cause of invasive *Aspergillus* infection among immunocompromised

patients [21, 22]. Invasive aspergillosis is often associated with high mortality; published data show that from 62% to higher than 85% of infections are fatal [21, 23, 24].

Chlorination is the required approach in United States for maintaining a disinfectant residual in drinking water distribution systems [25-27]. Free chlorine is the most frequently used disinfectant, and the use of monochloramine to maintain a disinfectant residual in treated drinking water is expected to increase [25, 26, 28]. Additionally, monochloramine is of growing interest and has been suggested as an effective secondary disinfectant for on-site premise plumbing disinfection [29-31]. However, the efficacy of free chlorine and monochloramine disinfection against waterborne fungi has not been well evaluated. An early study has demonstrated that waterborne fungi are resistant to chlorination but did not evaluate the disinfection kinetics because of limited data [32]. A previous study demonstrated species-dependent resistance to common water disinfection approaches by waterborne fungi, but did not evaluate the kinetics of disinfection [33]. Another study evaluated the free chlorine inactivation of waterborne fungi in settled surface water and estimated 60 mg•min/L of Ct (product of concentration C $\times$ contact time t) was needed to inactivate 80% fungi in the settled surface water [34]. However the Ct values estimation of this study was based on aimed initial disinfectant concentration but not measured real-time concentration, which could cause overestimation of the Ct values [34]. The current research regarding the disinfection efficiency of waterborne fungi is limited; there is still a lack of a detailed evaluation for the inactivation kinetics of waterborne fungi by free chlorine and monochloramine.

The present study was carried out to evaluate the free chlorine and monochloramine inactivation kinetics of type and clinical strains of *Aspergillus fumigatus*, as well as *Aspergillus versicolor* and *Penicillium purpurogenum* isolated from drinking water. These fungal species

have all previously been found in drinking water [1, 3, 16]. Disinfection of fungal spores was determined by culture in a laboratory study in phosphate buffered deionized water with the chlorine residual measured. Bayesian statistics based Markov Chain Monte Carlo method (MCMC) was used to fit observed data to the delayed Chick-Watson model, which is a modified version of the Chick-Watson model accounting for an initial lag phase of inactivation, to describe the disinfection kinetics based on experimental data [35-37]. Finally, observed disinfection kinetics were compared with values for other relevant drinking-water microorganisms.

5.2 MATERIALS AND METHODS

5.2.1 Fungal isolates and spore suspension preparation

An *Aspergillus fumigatus* type strain (ATCC 1022), *Aspergillus fumigatus* clinical isolate, *Aspergillus versicolor* tap water isolate, and *Penicillium purpurogenum* tap water isolate were each tested in the present study. The *Aspergillus fumigatus* clinical strain was kindly supplied by the Nguyen-Clancy Laboratory at University of Pittsburgh. *Aspergillus versicolor* and *Penicillium purpurogenum* were both isolated from laboratory cold tap water of Benedum Hall, University of Pittsburgh. For isolation, a 1 L water sample was filtered through a sterile 0.2 μm filter membrane (Pall) housed in a sterile filter funnel. The membranes were cultured on potato dextrose agar spiked with 100 mg/L chloramphenicol. Fungal colonies with light to dark green color were sub-cultured on potato dextrose agar. To confirm colony identity, DNA of the sub-cultured colonies was extracted using Powersoil DNA isolation kit following the manufacturer's

protocol. The ITS1 region of the extracted DNA was amplified using the previously described primer pair ITS1FI2 and ITS2 [38, 39]. PCR products were purified using Ampure XP beads (Beckman Coulter), and then sent for Sanger sequencing at the Genomic Research Core at University of Pittsburgh. The ITS1 sequences of isolated strains were compared against the UNITE database using BLASTn [40, 41], identifying the two isolated strains to be *Aspergillus* spp. and *Penicillium* spp. Species identifications were further confirmed with previously described morphological features and then sub-cultured for spore harvesting [42-44]. ITS1 amplicon sequences of the two tap water isolates are listed in Appendix C.

All tested fungal strains were sub-cultured on potato dextrose agar medium individually at 25ºC until sporulation. Spores were harvested by flooding the culture plates with 50 mL sterile 0.01 M PB buffered deionized water to form the original spore suspension solution. The original spore suspension was then washed three times with sterile 0.01 M PB buffered deionized water by centrifugation at 10,000×g for 3 minutes, and re-suspended in sterile 0.01 M PB buffered deionized water. The purified spore suspension was then stored in the dark at 4ºC for no more than two days before use.

5.2.2 Free chorine and monochloramine stock solution preparation

A commercially purchased 5% sodium hypochlorite solution (Acros Organics) was used as the free chlorine stock solution. A 10 mg/L monochloramine stock solution was prepared by mixing 5% sodium hypochlorite solution (Acros Organics) and 0.1 M ammonium chloride solution based on a Cl_2:N weight ratio of 3:1 in 0.01 M PB buffered deionized water (pH adjusted to 8) as previously described [33]. All stock solutions were freshly prepared before each inactivation experiment.

5.2.3 Free chlorine and monochloramine inactivation experiment

Before all experiments, glassware was soaked in a 0.5 g/L sodium hypochlorite solution overnight and washed with deionized water to remove any chlorine demand, and then autoclaved at 121℃ for 15 minutes. For the inactivation experiments, 5% sodium hypochlorite (Acros Organics) or freshly prepared 10 mg/L monochloramine stock solution was further diluted in 0.01 M PBS buffered DI water to achieve target concentration. Inactivation of *Aspergillus fumigatus* type strain and clinical strain were tested using target disinfectant concentrations of 1 mg/L and 4 mg/L. *Aspergillus versicolor* and *Penicillium purpurogenum* were tested using a target disinfectant concentration of 1 mg/L. Control experiments without disinfectant (0 mg/L) were conducted for all tested strains. All experiments were completed in duplicate.

Prior to experiments, the spore concentration of all tested fungal spore suspensions was enumerated by microscopy and normalized to approximately 6 $\log_{10}$ spores/mL, and then 3 mL of the normalized spore suspension was spiked into 147 mL of the disinfectant dilutions to achieve an initial spore concentration of approximately 4.3 $\log_{10}$ cells/mL. All spore suspensions were vortexed briefly before enumeration and spiking in the disinfectant solution. 5 mL samples for quantifying viable cells were collected at contact time 0, 5, 15, 30, 60 minutes, in a sterile tube containing 1 mL sterile 10% sodium thiosulfate solution to neutralize residual disinfectant. For *A. fumigatus* type strain and clinical strain inactivation experiments using 4 mg/L disinfectant concentration, samples were collected at contact time 0, 5, 10, 15, 30 minutes. Disinfectant concentration was monitored using a Hach colorimeter at each sampling time point following the manufacturer’s protocol. The Ct value at each sampling time point was determined as the area under the disinfectant residual vs. time curve (Appendix C Figure C1) as previously described [45, 46]. Flasks for the inactivation experiments were constantly stirred with a sterile

magnetic stir bar at 300 rpm. The pH was adjusted to 7.0 for free chlorine assays, and 8.0 for monochloramine assays according to described protocols [47], as these two pH levels were widely tested for free chlorine and monochloramine disinfection [33, 34]. All experiments were conducted at room temperature (22.5ºC). Temperature and pH were monitored during the experiment, and no change of temperature or pH was observed.

5.2.4 Estimation of disinfection kinetic parameters

To evaluate the disinfection kinetics, observed spore inactivation data were fit to the delayed Chick-Watson model, which is a modified version of the Chick-Watson model incorporating an initial lag phase [35-37]. A model fitting method based on Bayesian Markov Chain Monte Carlo (MCMC) derived from previous studies was used [28, 48]. The delayed Chick-Watson model considering random measurement errors is:

$$\ln\left(\frac{N}{N_0}\right)_i = \mu_i + \varepsilon_i, \qquad i = 1, \dots, n \qquad (1)$$

$$\mu_i = 0, \qquad when\ (Ct)_i \le Ct_{lag} \qquad (2)$$

$$\mu_i = k\left[(Ct)_i - Ct_{lag}\right], \qquad when\ (Ct)_i > Ct_{lag} \qquad (3)$$

Where N is the number of viable cells, N_0 is the initial number of viable cells at time 0, ε_i is the error with mean of 0 and variance σ^2, k is the inactivation rate constant, Ct is the product of disinfectant concentration (C) and contact time (t), and Ct_{lag} is the lag coefficient as previously described [36]. Equation (2) and (3) describe an initial lag phase followed by pseudo-first-order inactivation; if the Ct_{lag} equals zero the delayed Chick-Watson model is in the form of classical Chick-Watson model [28, 36]. The Bayesian MCMC estimation of model parameters was conducted using the software WinBUGS [49]. Six parallel chains were used for simulation; an initial simulation of 10,000 iterations was used as a burn-in phase, and 30,000 further iterations

were used to obtain the posterior distributions of model parameters. Trace plots and Gelman and Rubin diagnostics implemented in WinBUGS were used to determine the convergence of simulations. As previously described, a non-informative prior distribution was used for the MCMC estimation in this study [48]: diffuse inverse-gamma (0.001, 0.001) prior distribution was used for the variance σ^2; a normal distribution (0, 10^{-6}) was used as prior for rate constant k; in the present study, it is assumed that the lag coefficient Ct_{lag} can occur anywhere between Ct equals zero and the Ct value corresponding to the first observed cell inactivation, thus a uniform prior distribution between 0 and the Ct value corresponding to 10% of the maximum observed inactivation. Ct values for 2-log_{10} (99%), 3-log_{10} (99.9%), and 4-log_{10} (99.99%) inactivation were then calculated using a Bayesian MCMC method, based on the estimated disinfection kinetics parameters [48]. Detailed WinBUGS programming code is listed in Appendix C.

5.3 RESULTS AND DISCUSSION

5.3.1 Experimental overview

Duplicate free chlorine and monochloramine disinfection experiments were conducted for *A. fumigatus* type strain (ATCC 1022), A. fumigatus clinical strain, tap water isolate of *A. versicolor*, and *P. purpurogenum*. Control experiments without spiking any disinfectant (0 mg/L) were carried out for each tested fungal strain and disinfectant. No apparent inactivation was observed during all control experiments (data shown in Appendix C Figure C2), indicating there was no inactivation caused by the experimental set-up. The observed inactivation data demonstrated an apparent lag phase for the two *A. fumigatus* strains and the *P. purpurogenum*

tap water isolate (Figure 5.1 and 5.2). Numerous studies have demonstrated the delayed Chick-Watson model is appropriate for fitting disinfection data with an apparent lag phase [28, 48, 50, 51]. The delayed Chick-Watson model is also consistent with the Ct (concentration-time) concept, which is widely used to evaluate water disinfection efficacy against different microorganisms and used by USEPA to regulate water disinfection practices [28, 34, 52]. Thus the delayed Chick-Watson model was used to describe the disinfection kinetics in the present study. A classical Chick-Watson model without an initial lag phase was also used to fit observed disinfection data in this study (data not shown). By comparing the model predicted $Ln(N/N_0)$ against measured $Ln(N/N_0)$ at the same Ct values, the delayed Chick-Watson model demonstrated better fitting to linear relation with higher coefficients of determination (Appendix C Figure C3); therefore, in the present study the delayed Chick-Watson model was used to describe disinfection kinetics for all tested fungal strains.

5.3.2 Free chlorine and monochloramine disinfection kinetic parameters

Free chlorine disinfection experiments were conducted in a laboratory at room temperature (22.5ºC) and a pH of 7. Inactivation was observed for all tested fungi, but rates varied between isolates. At a free chlorine Ct of 60 mg•min/L, the observed inactivation level was 2.9-log_{10} (6.6-ln), 4.6-log_{10} (10.6-ln), 1.9-log_{10} (4.3-ln), and 0.9-log_{10} (2.2-ln) for *A. fumigatus* type strain and clinical strain, *A. versicolor*, and *P. purpurogenum*, respectively (Figure 5.1).

The delayed Chick-Watson model was applied to describe free chlorine disinfection kinetics (Figure 5.1). Both the *A. fumigatus* type and clinical strain demonstrated apparent lag phases prior to inactivation. The inactivation data demonstrated that the *A. fumigatus* type strain had a lag coefficient (Ct_{lag}) of 13.71 ± 2.04 mg•min/L, and *A. fumigatus* clinical strain had a lag

coefficient (Ct_{lag}) of 14.67 ± 5.66 mg•min/L. *A. versicolor* and *P. purpurogenum* demonstrated a shorter lag phase than the two *A. fumigatus* strains. Ct_{lag} of *A. versicolor* was 2.62 ± 1.15 mg•min/L, and Ct_{lag} of *P. purpurogenum* was 2.14 ± 1.22 mg•min/L (Table 5.1). The present study observed an apparent initial lag phase for the free chlorine inaction of *A. fumigatus* type strain and clinical strain, during which no apparent inactivation was occurred before pseudo-first order disinfection reaction. Shorter lag phase were estimated for *A. versicolor* and *P. purpurogenum*, with lower 95% credible bounds close to 0 (Table 5.1). This result indicates the presence of initial lag phase during fungal disinfection may be species-dependent. *A. fumigatus* showed a higher inactivation rate than the other two species, with rate constants (k) of -0.15 ± 0.01 L/mg•min and -0.22 ± 0.05 L/mg•min for type strain and clinical strain, respectively. Slower free chlorine inactivation rates were observed for *A. versicolor*, with rate constant (k) of -0.08 ± 0.007 L/mg•min. *P. purpurogenum* demonstrated the slowest free chlorine inactivation, with inactivation rate constant (k) of -0.04 ± 0.004 L/mg•min. A two sample t-test was conducted using Minitab indicated inactivation rate constants of each tested fungi are statistically significantly different (p-values < 0.05). This result demonstrates that inactivation rate constants of fungi varied between species, consistent with previous study [33]. Detailed free chlorine disinfection kinetic parameters estimations are listed in Table 5.1.

Monochloramine disinfection experiments were conducted in the laboratory at room temperature (22.5℃) and a pH of 8. At pH 8, monochloramine is the dominant chloramine species, thus the results are presented as monochloramine disinfection kinetics [28, 33]. At a monochloramine Ct of 60 mg•min/L, the observed inactivation level was 1.9-$\log_{10}$ (4.3-ln), 1.8-$\log_{10}$ (4.1-ln), 0.3-$\log_{10}$ (0.8-ln), and 1.1-$\log_{10}$ (2.5-ln) for *A. fumigatus* type strain and clinical strain, *A. versicolor*, and *P. purpurogenum*, respectively (Figure 5.2). A shorter lag phase was

observed during monochloramine disinfection reaction, except for *P. purpurogenum*. The experimental results demonstrated that *A. fumigatus* type and clinical strain, and *A. versicolor* had shorter initial lag phase during monochloramine disinfection comparing to free chlorine, with Ct_{lag} of 5.61 ± 2.11 mg•min/L, 1.41 ± 1.21 mg•min/L, and 1.85 ± 1.17 mg•min/L, respectively (Table 5.2). *P. purpurogenum* demonstrated a longer lag phase for monochloramine disinfection compared to free chlorine, with a Ct_{lag} of 10.91 ± 1.43 mg•min/L.

Previous studies suggested intrinsic resistance by cell wall acting as barrier for the entry of chemical agents contributes to fungal resistance to disinfection; and this resistance is linked to fungal cell wall porosity and thickness, which are affected by cell wall chemical composition [53]. The variation of cell wall porosity and thickness likely contributed to the variation of Ctlag among fungal isolates. Cell aggregation was also suggested as a potential cause of the initial lag phase during disinfection [54]. However, in the present study no apparent cell aggregation was observed during microscopic enumeration before spiking spore suspensions into disinfectant solutions, suggesting that cell aggregation was less likely to cause the initial lag phase.

For monochloramine disinfection, A. fumigatus also demonstrated the highest inactivation rate, with a rate constant of -0.07 ± 0.004 L/mg•min and -0.08 ± 0.006 L/mg•min for type strain and clinical strain, respectively. The inactivation rate constant of A. versicolor was -0.01 ± 0.002 L/mg•min and the inactivation rate constant of P. purpurogenum was -0.05 ± 0.003 L/mg•min. Similar to free chlorine inactivation, a statistically significant difference of inactivation rates among different fungal strains were observed for monochloramine (p-values < 0.05).

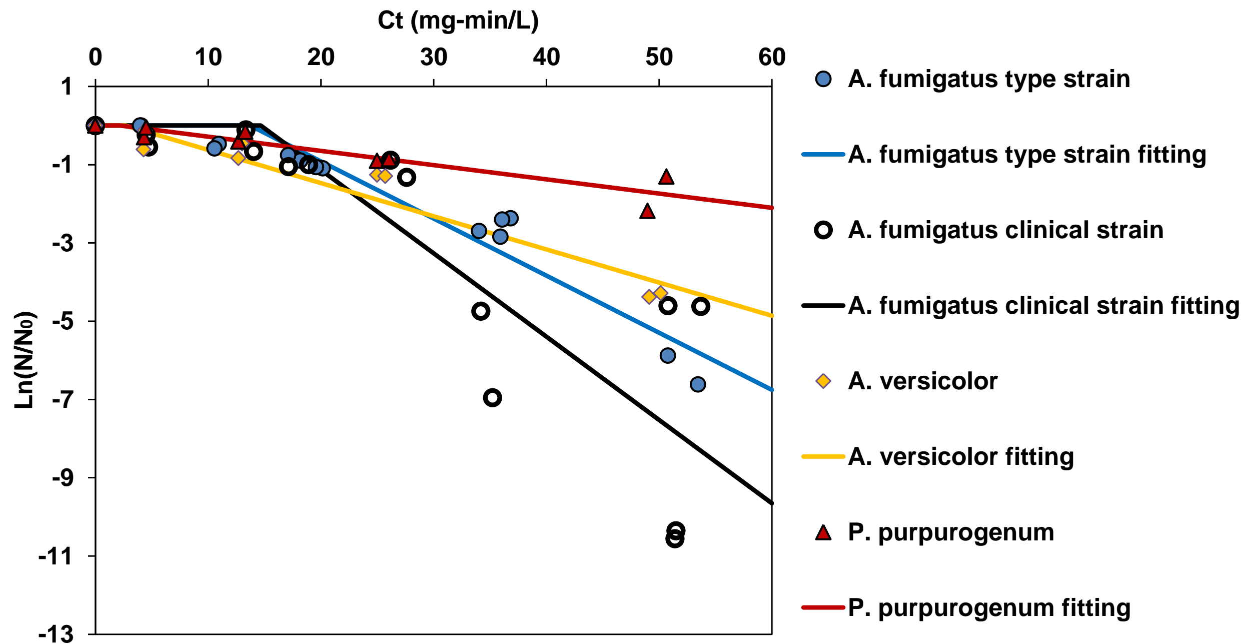

Figure 5.1 Free chlorine inactivation of tested fungal strains (pH = 7, temperature = 22.5ºC)

Table 5.1 Summary of inactivation kinetic parameters of free chlorine inactivation (pH = 7, temperature = 22.5ºC)

	k, mean±sd (L/mg·min)	k, 95% credible interval	Ct_{lag}, mean±sd (mg·min/L)	Ct_{lag}, 95% credible interval
A. fumigatus type strain	-0.15 ± 0.01	(-0.17, -0.12)	13.71 ± 2.04	(8.69, 16.73)
A. fumigatus clinical strain	-0.22 ± 0.05	(-0.32, -0.14)	14.67 ± 5.66	(3.02, 25.05)
A. versicolor	-0.08 ± 0.007	(-0.1, -0.07)	2.62 ± 1.15	(0.26, 4.21)
P. purpurogenum	-0.04 ± 0.004	(-0.04, -0.03)	2.14 ± 1.22	(0.11, 4.2)

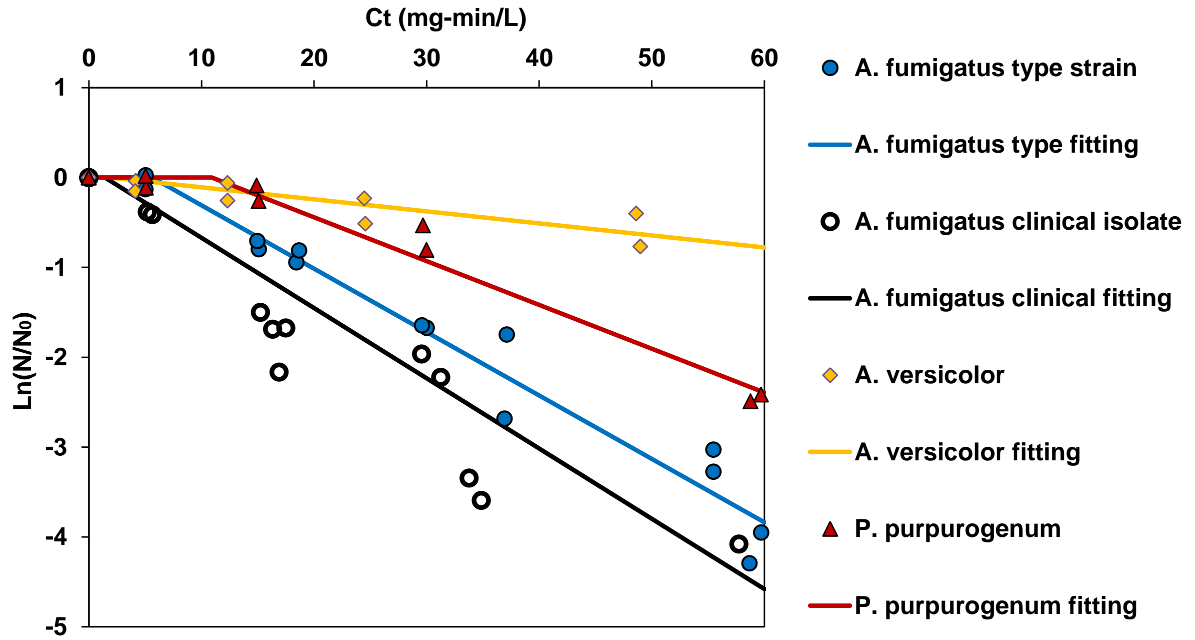

Figure 5.2 Monochloramine inactivation of tested fungal strains (pH = 8, temperature = 22.5°C)

Table 5.2 Summary of inactivation kinetic parameters of monochloramine inactivation (pH = 8, temperature = 22.5°C)

	k, mean±sd (L/mg·min)	k, 95% credible interval	Ct_{lag}, mean±sd (mg·min/L)	Ct_{lag}, 95% credible interval
A. fumigatus type strain	-0.07 ± 0.004	(-0.08, -0.07)	5.61 ± 2.11	(1.45, 9.75)
A. fumigatus clinical strain	-0.08 ± 0.006	(-0.09, -0.06)	1.41 ± 1.21	(0.04, 4.54)
A. versicolor	-0.01 ± 0.002	(-0.02, -0.009)	1.85 ± 1.17	(0.08, 3.97)
P. purpurogenum	-0.05 ± 0.003	(-0.05, -0.04)	10.91 ± 1.43	(7.05, 12.29)

For all free chlorine and monochloramine disinfection assays, the delayed Chick-Watson model predicted $Ln(N/N_0)$ were compared against the measured Ln(N/N0) at the same Ct levels (Figure 5.3 & 5.4). The model predicted spore survival ratio correlated to the measured survival ratio following linear relationship, with a slope ranging from 0.87 to 0.97 for free chlorine and ranging from 0.87 to 0.99 for monochloramine; the coefficients of determination (R2) ranged from 0.75 to 0.95 for free chlorine and 0.75 to 0.97 for monochloramine. Apparent outlier points, where measured $Ln(N/N_0)$ values were higher than predicted values, were observed for the A. fumigatus free chlorine inactivation, causing lower coefficient of determination comparing to other fungi (Figure 5.1 & 5.3). These outlier points were observed during inactivation experiment using 4 mg/L free chlorine; the more rapid cell die-off rate may contribute to the error during sample processing.

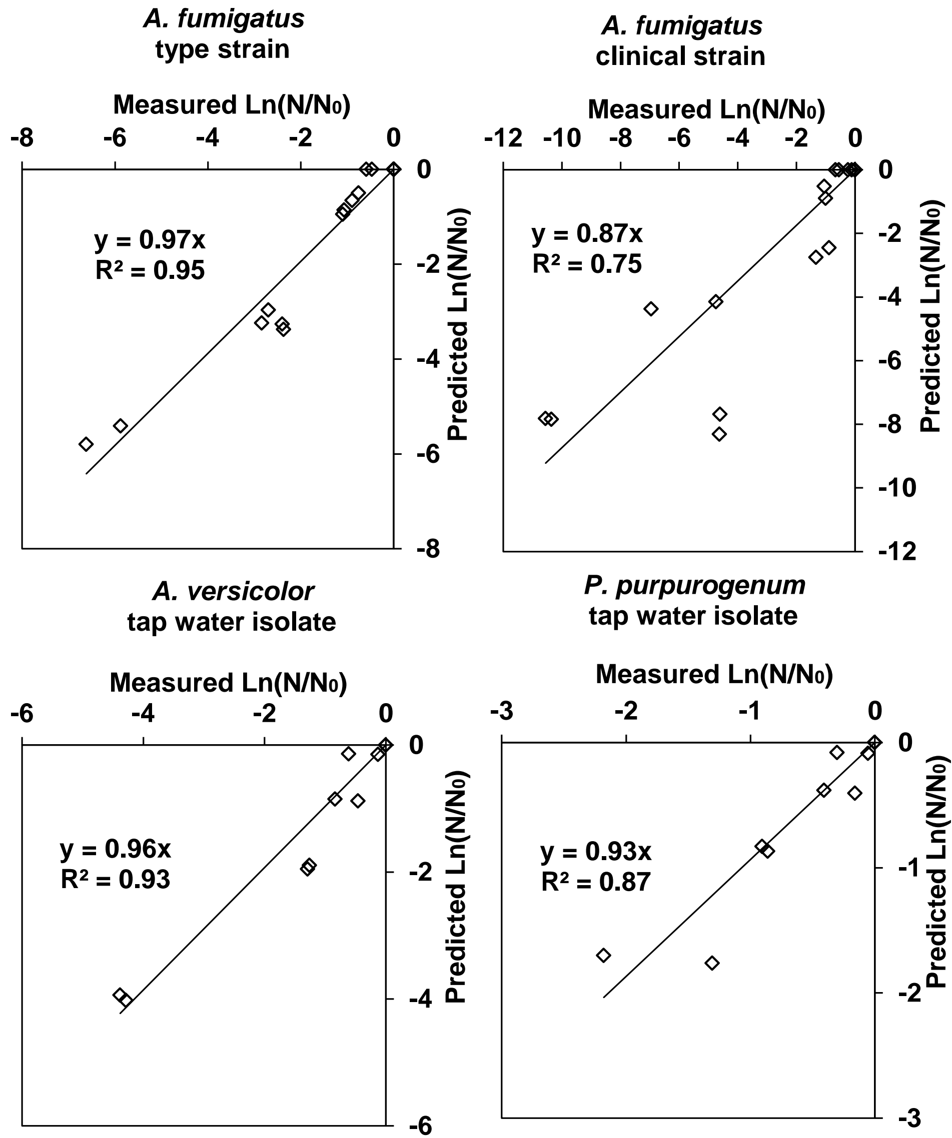

Figure 5.3 Comparison of delayed Chick-Watson model predicted $\mathrm{Ln}(N/N_0)$ and measured $\mathrm{Ln}(N/N_0)$ for free chlorine inactivation

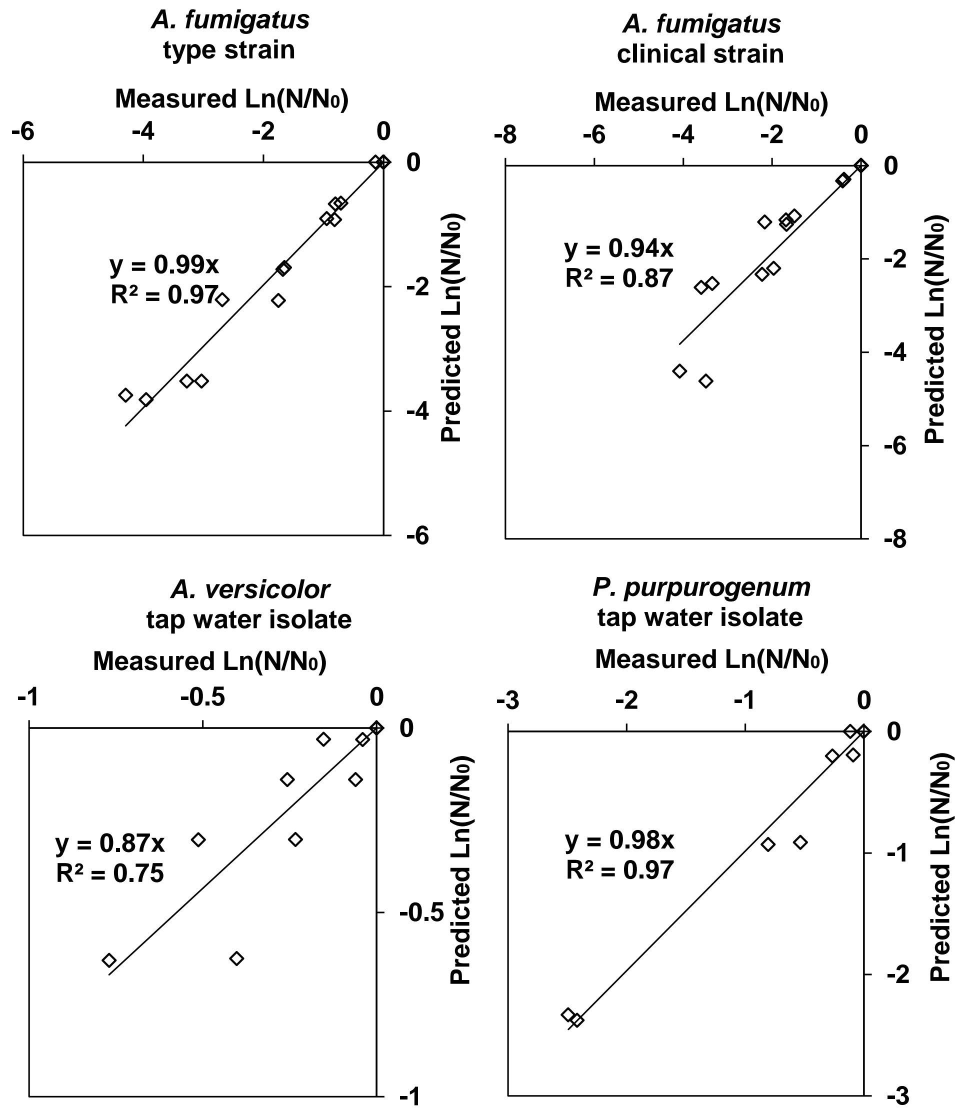

Figure 5.4 Comparison of delayed Chick-Watson model predicted Ln(N/N_0) and measured Ln(N/N_0) for monochloramine inactivation

5.3.3 Ct values for target inactivation levels

Required Ct values for 2-log_{10} (99%), 3-log_{10} (99.9%), and 4-log_{10} (99.99%) inactivation of each tested fungal isolate were calculated by using the disinfection kinetic parameters presented in the previous section. Under the tested conditions, P. purpurogenum demonstrated the highest required Ct values for target inactivation levels, followed by A. versicolor, and A. fumigatus. To achieve 3-log_{10} inactivation by free chlorine, the mean values of estimated Ct for A. fumigatus type strain, clinical strain, A. versicolor, and P. purpurogenum are 61.42 mg•min/L, 48.99 mg•min/L, 84.72 mg•min/L, and 194.7 mg•min/L, respectively. The required Ct values of 3-log_{10} monochloramine inactivation for A. fumigatus type strain, clinical strain, A. versicolor, and P. purpurogenum are 103.9 mg•min/L, 90.33 mg•min/L, 531.3 mg•min/L, and 153.2 mg•min/L, respectively. Detailed estimations including standard deviation and 95% credible bounds of required Ct values for target inactivating levels are listed in Table 5.3 and 5.4.

Figures 5.5 and 5.6 show required Ct values for 3-log_{10} inactivation of the tested fungi in the current study and other microorganisms derived from previous research. For both free chlorine and monochloramine, Ct values for 3-log_{10} inactivation of all tested fungi are higher than Adenovirus, *Escherichia coli*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae*, and within the similar range of Ct for 3-log_{10} inactivation of Mycobacterium spp. and *Legionella pneumophila* [55-60] (Figure 5.5 & 5.6). The current data show the tested fungi are generally resistant to free chlorine and monochloramine disinfection. For free chlorine, the Ct for 3-log10 inactivation of all the tested fungi are also higher than the USEPA recommended Ct for 3-log10 inactivation of Giardia, which is 37 mg•min/L [61]; while for monochloramine the USEPA recommended Ct for 3-log_{10} inactivation of Giardia is 1100 mg•min/L [61]. Further field-scale

evaluations are needed to assess disinfection efficacy of fungi in water treatment utilities following USEPA suggested disinfection conditions.

Table 5.3 Ct value (mg·min/L) estimation for 2-log_{10}, 3-log_{10}, and 4-log_{10} inactivation by free chlorine (pH = 7, temperature = 22.5ºC)

	$Ct_{2\text{-log}}$, mean ± sd	$Ct_{3\text{-log}}$, mean ± sd	$Ct_{4\text{-log}}$, mean ± sd
A. fumigatus type strain	45.52 ±3.506	61.42 ±4.741	77.33 ±6.06
A. fumigatus clinical strain	37.55 ±8.665	48.99 ±11.36	60.43 ±14.3
A. versicolor	57.35 ±4.648	84.72 ±6.853	112.1±9.081
P. purpurogenum	130.5 ±14.43	194.7 ±21.6	258.9 ±28.78

Table 5.4 Ct value (mg·min/L) estimation for 2-log_{10}, 3-log_{10}, and 4-log_{10} inactivation by monochloramine (pH = 8, temperature = 22.5ºC)

	$Ct_{2\text{-log}}$, mean ± sd	$Ct_{3\text{-log}}$, mean ± sd	$Ct_{4\text{-log}}$, mean ± sd
A. fumigatus type strain	71.15 ±4.702	103.9 ±6.644	136.7 ±8.661
A. fumigatus clinical strain	60.69 ±4.867	90.33 ±7.174	120 ±9.506
A. versicolor	354.8 ±58.08	531.3 ±87.11	707.8 ±116.1
P. purpurogenum	105.8 ±6.14	153.2 ±9.071	200.6 ±12.03

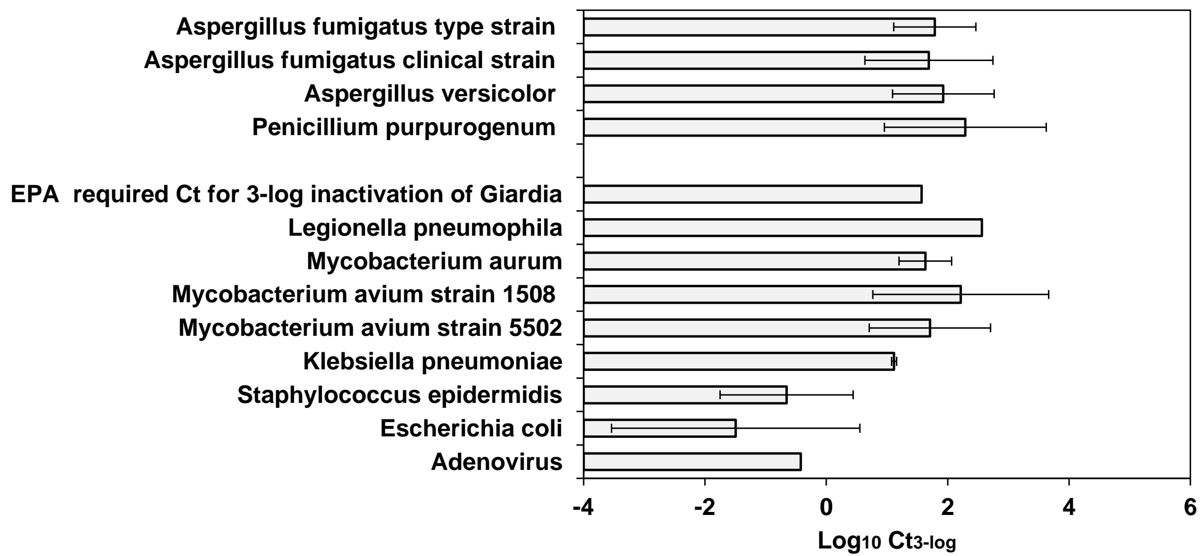

Figure 5.5 Comparison of free chlorine Ct values required for 3-log_{10} inactivation for the tested fungi and other microorganisms; 3-log_{10} Ct values for other microorganisms were directly derived or calculated based on inactivation rate constant from previous studies under similar experiment conditions (22.5°C, pH 7) [55-59, 61]

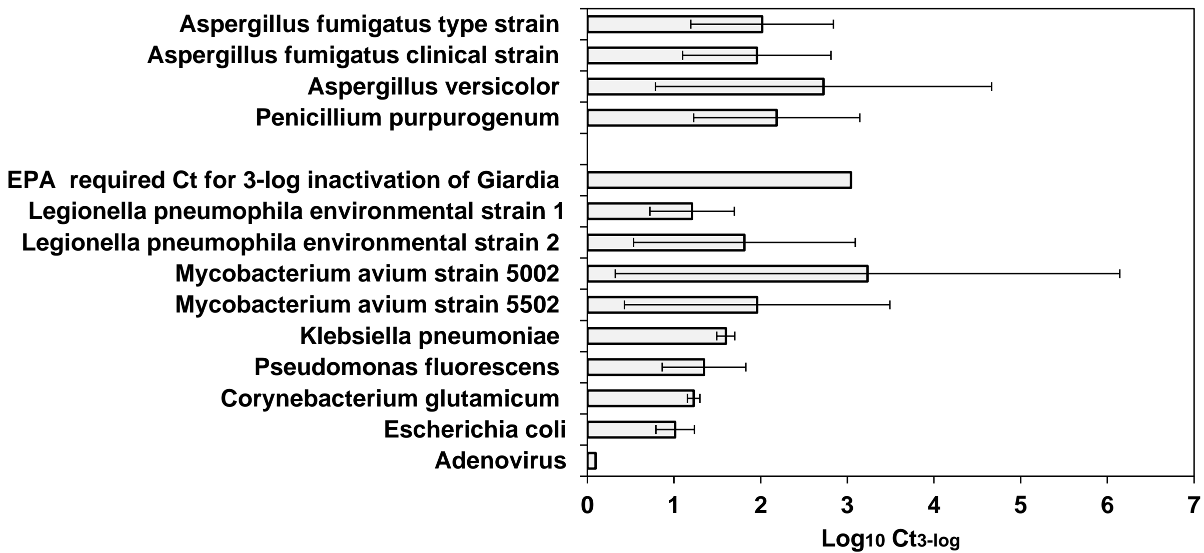

Figure 5.6 Comparison of monochloramine Ct values required for 3-log_{10} inactivation for the tested fungi and other microorganisms; 3-log_{10} Ct values for other microorganisms were directly derived or calculated based on inactivation rate constant from previous studies under similar experiment conditions (22.5°C, pH 8) [57-61]

5.4 SUMMARY AND CONCLUSIONS

This chapter presents the disinfection kinetics of *Aspergillus fumigatus*, *Aspergillus versicolor*, and *Penicillium purpurogenum* by free chlorine and monochloramine. An apparent initial lag phase was observed for *A. fumigatus* during free chlorine disinfection and *P. purpurogenum* during monochloramine disinfection. A delayed Chick-Watson model was used to fit the measured data and estimate the disinfection kinetic parameters. The results show *P. purpurogenum* was more resistant to free chlorine than *A. versicolor*, followed by *A. fumigatus*.

Regarding monochloramine disinfection, *A. versicolor* was the most resistant species, followed by *P. purpurogenum* and *A. fumigatus*. Resistance to free chlorine and monochloramine of the tested fungal strains, represented by Ct values for achieving 3-log_{10} inactivation, was found to be within a similar range of *Mycobacterium* spp. and *Legionella pneumophila*, which are known as disinfection resistant microorganisms. The current study shows that the tested fungi, including *Aspergillus fumigatus*, are resistant to free chlorine and monochloramine disinfection, potentially facilitating persistence and survival through drinking water treatment and distribution systems.

6.0 DISSERTATION SUMMARY AND CONCLUSIONS

6.1 SUMMARY

Drinking water treatment and distribution systems contain highly complex microbial communities, among which fungi are widely occurring microorganisms. Fungal ecology in drinking water systems has only been evaluated to a limited degree, hindering the holistic understanding of drinking water microbial ecology. Particularly, centralized drinking water treatment is the very beginning point of the whole drinking water system, and is critical to control the drinking water microbiological quality. Evidence shows centralized drinking water treatment processes shift waterborne bacterial community structure, but little is known regarding fungal community dynamics within this process. In addition, on-site secondary disinfection has been used to supplement disinfectant residual in premise plumbing system, and the knowledge of on-site disinfection's impact on microbial ecology is necessary for evaluating potential side effects but is currently limited. The objectives of this research were to: (1) review and summarize the current knowledge of fungal ecology in drinking water; (2) investigate fungal community dynamics within centralized drinking water treatment process; (3) investigate effect of premise plumbing on-site disinfection on fungal community structure; (4) evaluate the disinfection kinetics of common waterborne fungi.

In this research, a comprehensive literature review (Chapter 2) was conducted to summarize the current state of knowledge and identify needs for research regarding fungal ecology in drinking water. A field sampling study (Chapter 3) was conducted to evaluate fungal community structure dynamics within a conventional drinking water treatment process. The water treatment process posed a significant selection effect on the fungal community that was different from the observed effect on bacteria. Media filtration was found as the major threshold to shift fungal community structure regarding both diversity and absolute abundance. Study was carried out to investigate the effect of on-site secondary disinfection on fungal community structure dynamics (Chapter 4). This study found that the on-site disinfection did not shift the fungal community structure, and a diverse fungal community including potential opportunistic pathogens such as Aspergillus spp. was detected. The dissertation research also includes lab scale experiments to evaluate disinfection kinetics of *Aspergillus* and *Penicillium*, which are common waterborne fungal genera (Chapter 5). Detailed evaluation of free chlorine and monochloramine disinfection kinetics for waterborne fungal strains was conducted, and the tested fungal strains were found to be resistant to disinfection. Such data was unavailable before this study. Main findings of each study are summarized below:

6.1.1 Literature Review of Fungal Diversity in Drinking Water Systems

By comprehensively reviewing published literature regarding drinking water fungi, six fungal genera were found as the most commonly culture isolated fungi in drinking water, namely *Penicillium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Acremonium*, and *Trichoderma*. Published data suggests abundance of these genera ranges from 1 CFU/L to more than 3-$\log_{10}$ CFU/L. Due to previous methodological challenges and the limited amount of research conducted, knowledge

gaps were identified regarding the overall fungal diversity in drinking water, the effects of water treatment on fungal ecology, and the disinfection efficiency of fungi in drinking water.

6.1.2 Drinking Water Fungal Community Structure Shaped By Centralized Drinking Water Treatment Unit Operations

Within the studied full-scale drinking water treatment process, fungal abundance was significantly reduced after media filtration, but no further significant reduction was found after disinfection. Culturable fungi were isolated from post disinfection water. Both media filtration and disinfection significantly reduced bacterial abundance. The media filtration step also shaped the final fungal community structure - the number of fungal genera was reduced while the relative abundance of *Penicillium* and *Aspergillus* was found to increase in post media filtration water. Fungal community structure in post media filtration water and post disinfection water were similar to each other, indicating no significant community structure change during the disinfection step of water treatment.

6.1.3 Fungal Diversity in a Hospital Premise Plumbing System Treated with On-Site Monochloramine

A diverse fungal community, with 202 different fungal genera, was found in the studied hospital premise plumbing system. The core fungal biome was found to include *Penicillium*, *Aspergillus*, *Peniophora*, *Cladosporium*, and *Rhodosporidium* identified in more than 50% of samples. No significant change in community structure was found fungal community structure before and after on-site premise plumbing disinfection. Additionally, fungal genus level identification

accuracy by next-generation ITS amplicon sequencing ranged from 95.76% to 99.77%, consistent with previous research.

6.1.4 Inactivation Kinetics of *Aspergillus* and *Penicillium* by Free Chlorine and Monochloramine in Drinking Water

Free chlorine and monochloramine disinfection kinetics of *Aspergillus fumigatus*, *Aspergillus versicolor*, and *Penicillium purpurogenum* spores were evaluated. Initial lag phase during disinfection was found, potentially caused by an intrinsic fungal resistance to disinfectant. By fitting measured inactivation data to the delayed Chick-Watson model, which is consistent with Ct (concentration-time) concept, disinfection rate constants of tested fungi were found to range from -0.22 L/mg•min to -0.04 L/mg•min for free chlorine, and from -0.01 L/mg•min to -0.08 L/mg•min for monochloramine. The estimated Ct (disinfectant concentration × contact time) for 3-$\log_{10}$ inactivation of tested fungal strains ranged from 49.0 mg•min/L to 194.7 mg•min/L for free chlorine, and from 90.3 mg•min/L to 153.2 mg•min/L for monochloramine. The required Ct for 3-$\log_{10}$ inactivation of tested fungi were higher than previously evaluated virus such as Adenovirus and common waterborne bacteria, and within the similar range for 3-$\log_{10}$ inactivation of *Mycobacterium* spp. and *Legionella pneumophila*, indicating fungi are resistant to water disinfection using free chlorine or monochloramine.

6.2 CONCLUSIONS AND IMPLICATIONS FOR PRACTICE

The overall research conclusions from this dissertation work are:

1. **Fungi are common within the drinking water environment.** Studies using culture isolation widely found the occurrence of fungi in drinking water. Notable opportunistic pathogens, *Aspergillus fumigatus*, *Aspergillus terreus*, *Aspergillus flavus*, *Fusarium solani*, and *Fusarium oxysporum*, were frequently found within the spectrum of drinking water fungi, indicating the drinking water could be an environmental source of pathogenic fungi. Further field-scale investigation regarding presence and transmission mechanisms of waterborne opportunistic fungal pathogens could benefit further reducing opportunistic infectious risks in facilities with concerns for nosocomial fungal infections.

2. **Media filtration within centralized drinking water treatment process is the primary threshold to control fungal abundance and community structure.** The study conducted in a full scale drinking water treatment process demonstrates that media filtration significantly reduced fungal abundance and acted as the threshold to shape fungal community structure in drinking water. Combined with the findings that disinfection does not pose a significant effect on fungal community structure, it indicates media filtration is the primary factor affecting fungal ecology in drinking water.

3. **Waterborne fungi are resistant to free chlorine and monochloramine disinfection.** Results from Chapter 3 and 4 demonstrate that water disinfection did not significantly affect fungal community structure. Chapter 5 demonstrates that common waterborne fungi such as *Penicillium* and *Aspergillus* are resistant to water disinfection by free chlorine and monochloramine, which

could facilitate the survival of fungi in treated drinking water. Further field-scale studies are needed to evaluate disinfection efficacy of fungi in full scale water treatment utilities.

6.3 KEY CONTRIBUTIONS

This study highlighted that the physical processes, such as sedimentation and media filtration, within centralized drinking water treatment significantly shifted the fungal community structure, while free chlorine disinfection as well as on-site monochloramine disinfection did not pose significant effect on the drinking water fungal community structure. This study also highlighted the significant increase of relative abundance of *Penicillium* along the centralized drinking water treatment processes. The selection effect towards *Penicillium* by centralized water treatment could explain its dominance in treated drinking water. The shifting of fungal and bacterial community structure in correlation with centralized drinking water treatment processes highlighted in this study also indicates possibilities to artificially manage drinking water microbiome. The disinfection kinetics of *Penicillium* and *Aspergillus* by free chlorine and monochloramine highlighted in this study could be used by water industry to further evaluate disinfection processes' efficiency to remove fungi from water.

APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 3

A.1 Heterotrophic plate count and total filamentous fungal colony count

Heterotrophic plate count (HPC) analysis was conducted to evaluate the total culturable heterotrophic bacterial abundance through the water treatment processes train. 1 mL original water sample was plated by pour-plate method using Difco plate count agar (BD), 0.1 mL ten-fold serial dilutions (10^{-1} to 10^{-4}) of the original sample were plated by spread-plate method using Difco plate count agar (BD). Plating of each original sample and serial dilution was conducted in triplicate. All plates were incubated at 37°C for 48 hours before analysis. Enumeration of heterotrophic plate counts was based on the average of highest countable dilution plated, and presented as colony forming units (CFU)/mL. The lower detection limit was 1 CFU/mL. During each experiment, one agar plate without plating anything and one agar plate plated with 1 mL sterile deionized water used for serial dilution were included as negative controls. All negative controls showed negative.

Total filamentous fungal colony count analysis was conducted to evaluate the total culturable filamentous fungal abundance through the water treatment processes train. 1 mL, 10 mL, 100 mL, and 1 L of each original water sample was filtered in duplicates through 0.2 μm

sterile Supor® 200 Polyethersulfone membranes (Pall Corporation) housed in sterile Nalgene analytical filter funnels (Thermo Scientific; Fisher). The filter membranes were then directly plated on potato dextrose agar (DB) supplemented with 100 mg/L of chloramphenicol. All plates were incubated at 25°C for two weeks and checked every 2 days during incubation. Enumeration of total filamentous fungal colony counts was based on the average of highest volume of sample filtered and plated, and presented as colony forming units (CFU)/L. The lower detection limit was 1 CFU/L. During each experiment, one agar plate without plating anything and one agar plate plated with new filter membrane were included as negative controls. All negative controls showed negative.

A.2 ITS1 PCR

Triplicate PCR reactions were performed in 20 µL reaction mixtures containing 1 µL of sample DNA, 10 µL of 2x DreamTaq Master Mix polymerase (Thermo Scientific), and 0.5 µL of each 10 µM primer. Temperature condition was 15 minutes denaturation at 95°C, followed by 40 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds extension at 72°C, and a final elongation at 72°C for 5 minutes.

A.3 16S rRNA PCR

Duplicate PCR reactions were performed in 25 µL reaction mixtures containing 1 µL of sample DNA, 12.5 µL of 2x DreamTaq Master Mix polymerase (Thermo Scientific), and 0.2 µL of each 10 µM primer. Temperature condition was 3 minutes denaturation at 96°C, followed by 40 cycles of 45 seconds denaturation at 96°C, 1 minute annealing at 50°C, and 1 minute extension at 72°C, and a final elongation at 72°C for 10 minutes.

A.4 qPCR

All qPCR reactions were performed in triplicate. Each reaction was in 20 µL of total volume, included 1 µL of sample DNA, 10 µL of SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), and 0.5 µL of each 10 µM primer. All reactions were carried out on CFX Connect Real-Time PCR system (Bio Rad), temperature condition for fungal qPCR was 3 minutes initial denaturation at 98°C, followed by 40 cycles of 15 seconds denaturation at 98°C and 1 minute annealing and extension at 60°C. The temperature condition for both bacterial qPCR and *Acanthamoeba* spp. qPCR was 3 minutes initial denaturation at 98°C, followed by 40 cycles of 10 seconds denaturation at 98°C and 30 seconds annealing and extension at 60°C. Melt-curve analysis was conducted with 5 seconds at 65°C and 5 seconds at 95°C ensured samples and standard curve had same melt curve values.

A.5 *Aspergillus fumigatus* alkaline protease gene PCR

Duplicate PCR was conducted in 20 µL of total volume including 1 µL of sample DNA, 0.5 µL of each primer (10 mM), and 10 µL 2X DreamTaq PCR Master Mix (Thermo Fisher Scientific). The thermo cycling condition was 95ºC for 3 minutes, followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 1 minute, and 72ºC for 1 minute and final elongation at 72ºC for 5 minutes. The PCR results were analyzed on 2% agarose gel stained by Sybr Safe DNA gel stain (Thermo Fisher Scientific).

Table A1 Statistics of trimmed sequencing results

	Total number of sequences	Average number of sequences per sample	Standard deviation	Median sequence length (bp)
ITS1	446902	7326	7866	251
16S	548098	17128	6181	251

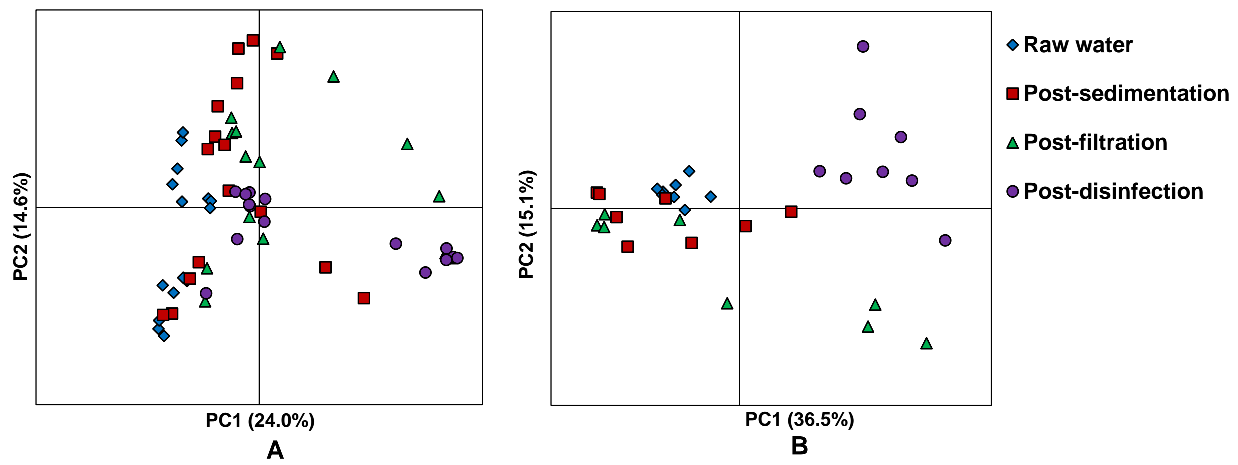

Figure A1 PCoA plots based on (A) fungal Bray-Curtis dissimilarity matrix and (B) bacterial weighted UniFrac dissimilarity matrix

Table A2 Pairwise ADONIS analysis results of fungal community

Based on Jaccard distance			
	RW	P-S	P-F
P-S	**0.001** (0.115)		
P-F	**0.001** (0.184)	**0.001** (0.075)	
P-D	**0.001** (0.194)	**0.001** (0.083)	0.225 (0.038)
Based on Bray-Curtis distance			
	RW	P-S	P-F
P-S	**0.003** (0.093)		
P-F	**0.001** (0.184)	**0.015** (0.066)	
P-D	**0.001** (0.217)	**0.002** (0.112)	0.115 (0.054)

Significant p-values are shown in bold ($p < 0.05$)

Table A3 Pairwise ADONIS analysis results of bacterial community

Unweighted UniFrac			
	RW	P-S	P-F
P-S	**0.001** (0.146)		
P-F	**0.001** (0.247)	**0.006** (0.122)	
P-D	**0.001** (0.357)	**0.001** (0.285)	**0.001** (0.199)
Weighted UniFrac			
	RW	P-S	P-F
P-S	**0.017** (0.153)		
P-F	**0.001** (0.220)	0.13 (0.120)	
P-D	**0.001** (0.388)	**0.001** (0.365)	**0.001** (0.258)

Significant p-values are shown in bold ($p < 0.05$)

APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 4

B.1 Complete sequence of primers

All primers written 5’-3’.

Complete sequence of forward primer (Illumina forward adapter, primer pad, primer linker, ITS1-FI2):

AATGATACGGCGACCACCGAGATCTACAC-TATGGTAATT-GT-GAACCWGCGGARGGATCA.

Complete sequence of reverse primer (Illumina reverse adapter, Golay barcode, primer pad, primer linker, ITS2):

CAAGCAGAAGACGGCATACGAGAT-NNNNNNNNNNNN-AGTCAGTCAG-CC-GCTGCGTTCTTCATCGATGC

Complete sequence of customized read 1 primer:

TATGGTAATT GTGAACCWGCGGARGGATCA

Complete sequence of customized read 2 primer:

AGTCAGTCAGCCGCTGCGTTCTTCATCGATGC

Complete sequence of customized index primer:

GCATCGATGAAGAACGCAGCGGCTGACTGACT

B.2 PCR condition and sequencing procedure

PCR reactions were performed in 20 µL reaction mixtures containing 1 µL of sample DNA, 10 µL of 2x DreamTaq Master Mix polymerase (Thermo Scientific), and 0.25 µM of each of the combinatorial ITS1FI2 and ITS2 primers. Temperature condition was 15 minutes denaturation at 95°C, followed by 40 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds extension at 72°C, and a final elongation at 72°C for 5 minutes. PCR reaction for each sample includes three replicates, and replicates of each reaction were pooled after PCR amplification and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). Negative controls in which the template DNA was replaced with 1 uL sterile molecular biology grade water were included in each PCR run. Gel visualization of AMPure XP beads purified PCR products were performed for quality inspection.

The purified PCR products of environmental samples were quantified by Qubit 2.0 Fluorometer with Qubit dsDNA HS Assay Kit (Invitrogen), and then normalized and pooled based on equal molarity. One additional purification was performed for the final pool of PCR products from environmental samples with Agencourt AMPure XP magnetic beads. Then the purified library was loaded to Illumina MiSeq Desktop Sequencer (Illumina) for sequencing by using customized read 1, read 2, and index primers.

For PCR products of pure culture fungal DNA, the purified products were purified by gel extraction again, then normalized and pooled based on equal molarity to form the final pure culture sequencing library and loaded to Illumina MiSeq Desktop Sequencer using the same customized sequencing primers described above in another sequencing run.

Table B1 Teleomorphs trimmed from reference database

Sequence ID	Fungal name	
GU981618	*Eupenicillium*	*abidjanum*
GU981617	*Eupenicillium*	*reticulisporum*
GU981616	*Eupenicillium*	*meridianum*
GU981615	*Eupenicillium*	*brefeldianum*
GU981614	*Eupenicillium*	*javanicum*
GU981613	*Eupenicillium*	*javanicum*
GU981607	*Eupenicillium*	*levitum*
GU981605	*Eupenicillium*	*Javanicum var melofor*
GU981582	*Eupenicillium*	*abidjanum*
GU981581	*Eupenicillium*	*zonatum*
GU981580	*Eupenicillium*	*brefeldianum*
GU981579	*Eupenicillium*	*javanicum var lineola*
GU981578	*Eupenicillium*	*ehrlichii*
GU981568	*Eupenicillium*	*limosum*
AB369900	*Neosartorya*	*fischeri*
AY354240	*Eupenicillium*	*pinetorum*
AY373895	*Neosartorya*	*fischeri*
AY373894	*Neosartorya*	*fischeri*
AM992114	*Eupenicillium*	*meridianum*
AM992113	*Eupenicillium*	*crustaceum*
AM992112	*Eupenicillium*	*baarnense*
AM992111	*Eupenicillium*	*terrenum*
HM036591	*Eupenicillium*	*idahoense*
GU966506	*Eupenicillium*	*javanicum*
GU966493	*Neosartorya*	*fischeri*
GQ221148	*Eupenicillium*	*molle*
GQ461906	*Neosartorya*	*hiratsukae*
FJ231014	*Eupenicillium*	*hirayamae*
FJ231013	*Eupenicillium*	*hirayamae*
FJ624264	*Neosartorya*	*fischeri*

Table B1 (Continued)

EF669995	*Neosartorya*	*fennelliae*
EF669994	*Neosartorya*	*fennelliae*
EF669988	*Neosartorya*	*spinosa*
EF669984	*Neosartorya*	*stramenia*
EF669983	*Neosartorya*	*fischeri*
EF669982	*Neosartorya*	*tatenoi*
EF669980	*Neosartorya*	*aurata*
EF669979	*Neosartorya*	*aurata*
EF669976	*Neosartorya*	*quadricincta*
EF669975	*Neosartorya*	*fischeri*
EF669973	*Neosartorya*	*spinosa*
EF669972	*Neosartorya*	*fischeri*
EF669966	*Neosartorya*	*pseudofischeri*
EF669965	*Neosartorya*	*spinosa*
EF669964	*Neosartorya*	*glabra*
EF669963	*Neosartorya*	*quadricincta*
EF669962	*Neosartorya*	*otanii*
EF669961	*Neosartorya*	*otanii*
EF669960	*Neosartorya*	*fennelliae*
EF669959	*Neosartorya*	*spinosa*
EF669955	*Neosartorya*	*spinosa*
EF669952	*Neosartorya*	*aureola*
EF669950	*Neosartorya*	*aureola*
EF669949	*Neosartorya*	*quadricincta*
EF669948	*Neosartorya*	*glabra*
EF669947	*Neosartorya*	*quadricincta*
EF669946	*Neosartorya*	*pseudofischeri*
EF669945	*Neosartorya*	*aureola*
EF669944	*Neosartorya*	*spathulata*
EF669943	*Neosartorya*	*spathulata*
EF669941	*Neosartorya*	*pseudofischeri*
EF669940	*Neosartorya*	*spinosa*
EF669939	*Neosartorya*	*pseudofischeri*
EF669938	*Neosartorya*	*glabra*
EF669937	*Neosartorya*	*pseudofischeri*
EF669936	*Neosartorya*	*fischeri*
EF669935	*Neosartorya*	*pseudofischeri*
EF422844	*Eupenicillium*	*cinnamopurpureum*
EF422843	*Eupenicillium*	*cinnamopurpureum*
EF422842	*Eupenicillium*	*cinnamopurpureum*
EF422841	*Eupenicillium*	*cinnamopurpureum*

Table B1 (Continued)

DQ494437	*Eupenicillium*	*pinetorum*
DQ494435	*Eupenicillium*	*pinetorum*
AF459733	*Neosartorya*	*stramenia*
AF459730	*Neosartorya*	*quadricincta*
AF459729	*Neosartorya*	*pseudofischeri*
EU427295	*Eupenicillium*	*osmophilum*
EU427292	*Eupenicillium*	*tropicum*
EU427289	*Eupenicillium*	*erubescens*
EU427286	*Eupenicillium*	*zonatum*
EU427284	*Eupenicillium*	*cinnamopurpureum*
EF626961	*Eupenicillium*	*ochrosalmoneum*
EF626960	*Eupenicillium*	*ochrosalmoneum*
EF626959	*Eupenicillium*	*ochrosalmoneum*
EF626958	*Eupenicillium*	*ochrosalmoneum*
EF626955	*Eupenicillium*	*idahoense*
AF033462	*Eupenicillium*	*rubidurum*
AF033454	*Eupenicillium*	*alutaceum*
AF033435	*Eupenicillium*	*brefeldianum*
AF033431	*Eupenicillium*	*inusitatum*
AF033430	*Eupenicillium*	*lassenii*
AF033425	*Eupenicillium*	*anatolicum*
AF033414	*Eupenicillium*	*cinnamopurpureum*
AF033409	*Eupenicillium*	*lapidosum*
GQ924907	*Eupenicillium*	*shearii*
FJ527878	*Eupenicillium*	*brefeldianum*
EU715611	*Neosartorya*	*hiratsukae*
U18358	*Eupenicillium*	*javanicum*
U18355	*Neosartorya*	*fischeri*
EU714322	*Neosartorya*	*hiratsukae*
EU926976	*Neosartorya*	*fischeri*
EU622253	*Pleurotus*	*ostreatus*
EU593904	*Neosartorya*	*hiratsukae*
EU551199	*Neosartorya*	*fischeri*
EU543210	*Neosartorya*	*hiratsukae*
EU515147	*Neosartorya*	*hiratsukae*
EU030360	*Eupenicillium*	*lapidosum*
EF531696	*Eupenicillium*	*anatolicum*
EF488446	*Eupenicillium*	*brefeldianum*
DQ536524	*Eupenicillium*	*parvum*
DQ473558	*Eupenicillium*	*pinetorum*
DQ401533	*Neosartorya*	*fischeri*

Table B1 (Continued)

DQ314733	*Macrophomina*	*phaseolina*
AY330710	*Neosartorya*	*pseudofischeri*
AY213647	*Eupenicillium*	*cinnamopurpureum*
AY232278	*Eupenicillium*	*shearii*
AF455541	*Neosartorya*	*fischeri*
AF455538	*Neosartorya*	*fischeri*
AF176661	*Neosartorya*	*fischeri*
AF263347	*Eupenicillium*	*bovifimosum*
AF033487	*Eupenicillium*	*tularense*
AF033481	*Eupenicillium*	*baarnense*
AF033467	*Eupenicillium*	*egyptiacum*
AF033466	*Eupenicillium*	*crustaceum*
AF033464	*Eupenicillium*	*erubescens*
AF033458	*Eupenicillium*	*katangense*
AF033446	*Eupenicillium*	*terrenum*
AF033444	*Eupenicillium*	*stolkiae*
AF033437	*Eupenicillium*	*reticulisporum*
AF033436	*Eupenicillium*	*levitum*
AF033432	*Eupenicillium*	*ehrlichii*
AF033420	*Eupenicillium*	*shearii*
AF033418	*Eupenicillium*	*hirayamae*
AF033411	*Eupenicillium*	*pinetorum*
AB479321	*Eupenicillium*	*crustaceum*
AB479320	*Eupenicillium*	*molle*
AB479319	*Eupenicillium*	*egyptiacum*
AB470908	*Bionectria*	*ochroleuca*
AB185272	*Neosartorya*	*glabra*
AB185271	*Neosartorya*	*spinosa*
AB185270	*Neosartorya*	*glabra*
AB185269	*Neosartorya*	*tatenoi*
AB185268	*Neosartorya*	*tatenoi*
AB185267	*Neosartorya*	*spathulata*
AB185266	*Neosartorya*	*aurata*
AB185265	*Neosartorya*	*udagawae*
AB185264	*Neosartorya*	*aureola*
AB185263	*Neosartorya*	*stramenia*
AB185262	*Neosartorya*	*fennelliae*
AB185261	*Neosartorya*	*primulina*
AB185260	*Neosartorya*	*quadricincta*
AB185259	*Neosartorya*	*botucatensis*
AB185258	*Neosartorya*	*spinosa*

Table B1 (Continued)

AB185257	*Neosartorya*	*hiratsukae*
AB185256	*Neosartorya*	*pseudofischeri*
AB185255	*Neosartorya*	*glabra*
AB185254	*Neosartorya*	*fischeri*
AB299414	*Neosartorya*	*coreana*
AB299413	*Neosartorya*	*laciniosa*
AB250782	*Neosartorya*	*udagawae*
AB250781	*Neosartorya*	*udagawae*
AJ004893	*Eupenicillium*	*shearii*
AJ004892	*Eupenicillium*	*crustaceum*
HQ407424	*Eupenicillium*	*brefeldianum*
HQ129858	*Eupenicillium*	*brefeldianum*
FJ004324	*Eupenicillium*	*cinnamopurpureum*
GU046487	*Hypocrea*	*lixii*
HQ166710	*Neosartorya*	*fischeri*
FR733875	*Neosartorya*	*laciniosa*
FR733873	*Neosartorya*	*hiratsukae*
FR733872	*Neosartorya*	*hiratsukae*
FR733871	*Neosartorya*	*quadricincta*
FR837959	*Neosartorya*	*hiratsukae*
HQ263372	*Neosartorya*	*udagawae*
HQ263364	*Neosartorya*	*udagawae*
HQ263363	*Neosartorya*	*udagawae*
HQ263362	*Neosartorya*	*udagawae*
HQ263361	*Neosartorya*	*udagawae*
HQ710545	*Eupenicillium*	*javanicum*
HQ608058	*Eupenicillium*	*rubidurum*
HQ607997	*Neosartorya*	*fischeri*
HQ607978	*Eupenicillium*	*rubidurum*
JN089772	*Eupenicillium*	*ochrosalmoneum*
JN093268	*Neosartorya*	*aureola*
JN114420	*Ustilago*	*tritici*
EU982016	*Neosartorya*	*fischeri*
JN564000	*Neosartorya*	*fennelliae*
JN390830	*Neosartorya*	*fischeri*
HE578066	*Neosartorya*	*hiratsukae*
HE578061	*Neosartorya*	*udagawae*
JN252116	*Hypocrea*	*lixii*
JN252106	*Hypocrea*	*lixii*
JN252105	*Hypocrea*	*lixii*
JN943591	*Neosartorya*	*udagawae*

Table B1 (Continued)

JN943589	*Neosartorya*	*spinosa*
JN943587	*Neosartorya*	*spathulata*
JN943585	*Neosartorya*	*quadricincta*
JN943583	*Neosartorya*	*pseudofischeri*
JN943581	*Neosartorya*	*laciniosa*
JN943579	*Neosartorya*	*hiratsukae*
JN943577	*Neosartorya*	*glabra*
JN943575	*Neosartorya*	*fischeri*
JN943573	*Neosartorya*	*fennelliae*
JN943571	*Neosartorya*	*fennelliae*
JN943569	*Neosartorya*	*coreana*
JN943592	*Neosartorya*	*udagawae*
JN943590	*Neosartorya*	*spinosa*
JN943588	*Neosartorya*	*spathulata*
JN943586	*Neosartorya*	*quadricincta*
JN943584	*Neosartorya*	*pseudofischeri*
JN943582	*Neosartorya*	*laciniosa*
JN943580	*Neosartorya*	*hiratsukae*
JN943578	*Neosartorya*	*glabra*
JN943576	*Neosartorya*	*fischeri*
JN943574	*Neosartorya*	*fennelliae*
JN943572	*Neosartorya*	*fennelliae*
JN943570	*Neosartorya*	*coreana*
JF922037	*Neosartorya*	*fischeri*
JF922036	*Eupenicillium*	*osmophilum*
JQ680034	*Eupenicillium*	*brefeldianum*
HE974448	*Neosartorya*	*nishimurae*
HE974446	*Neosartorya*	*tsunodae*
HE974444	*Neosartorya*	*multiplicata*
HE974449	*Neosartorya*	*nishimurae*
HE974447	*Neosartorya*	*tsunodae*
HE974445	*Neosartorya*	*multiplicata*
JQ316519	*Neosartorya*	*udagawae*

Table B2 Accuracy of pure culture fungal DNA taxonomy identification on genus level

Tested fungi	Assigned taxa	Number of sequences	True/False	TIR (%)	FIR (%)	ER (%)
Alternaria alternata	*Alternaria*	972	T	96.91	0.10	0.10
	Cladosporium	1	F			
	Ambiguous	30	U/D			
	Total	1003				
Aspergillus fumigatus	*Aspergillus*	2517	T	99.76	0.08	0.08
	Cladosporium	1	F			
	Epicoccum	1	F			
	Ambiguous	4	U/D			
	Total	2523				
Cladosporium cladosporioides	*Cladosporium*	1560	T	95.76	0.18	0.19
	Omphalotus	1	F			
	Aspergillus	1	F			
	Talaromyces	1	F			
	Ambiguous	66	U/D			
	Total	1629				
Epicoccum nigrum	*Epicoccum*	1289	T	99.77	0.08	0.08
	Cladosporium	1	F			
	Ambiguous	2	U/D			
	Total	1292				
Penicillium chrysogenum	*Penicillium*	1782	T	99.61	0.06	0.06
	Alternaria	1	F			
	Ambiguous	6	U/D			
	Total	1789				

N/D: Not determined because of ambiguous identity at this taxonomic rank;
TIR (true identification ratio) = N_{true}/N_{total};
FIR (false identification ratio) = N_{false}/N_{total};
ER (error ratio) = $N_{false}/(N_{true}+N_{false})$;
N_{true}: number of sequences assigned to correct taxonomy. N_{false}: number of sequences assigned to false taxonomy. N_{total}: total number of sequences [1]

Table B3 Accuracy of pure culture fungal DNA taxonomy identification on species level

Tested fungi	Assigned taxa	Number of sequences	True/ False	TIR (%)	FIR (%)	ER (%)
Alternaria alternata	*Alternaria alternata*	14	T	1.40	1.20	46.15
	Alternaria compacta	3	F			
	Alternaria mali	4	F			
	Alternaria tenuissima	3	F			
	Alternaria porri	1	F			
	Alternaria spp.	947	U/D			
	*Cladosporium*spp.	1	F			
	Ambiguous	30	U/D			
	Total	1003				
Aspergillus fumigatus	*Aspergillus fumigatus*	20	T	0.79	0.08	9.09
	Aspergillus spp.	2497	U/D			
	Epicoccum nigrum	1	F			
	*Cladosporium*spp.	1	F			
	Ambiguous	4	U/D			
	Total	2523				
Cladosporium cladosporioides	*Cladosporium cladosporioides*	11	T	0.68	0.18	21.43
	*Cladosporium*spp.	1549	U/D			
	Talaromyces wortmannii	1	F			
	Omphalotus olearius	1	F			
	Aspergillus versicolor	1	F			
	Ambiguous	66	U/D			
	Total	1629				
Epicoccum nigrum	*Epicoccum nigrum*	1289	T	99.77	0.08	0.08
	*Cladosporium*spp.	1	F			
	Ambiguous	2	U/D			
	Total	1292				
Penicillium chrysogenum	*Penicillium chrysogenum*	7	T	0.39	0.50	56.25
	Penicillium coprobium	1	F			
	Penicillium granulatum	5	F			
	Penicillium commune	1	F			
	Penicillium italicum	1	F			
	Penicillium spp.	1767	U/D			
	Alternaria spp.	1	F			
	Ambiguous	6	U/D			

Table B3 (Continued)

Total	1789

N/D: Not determined because of ambiguous identity at this taxonomic rank
TIR (true identification ratio) = N_{true}/N_{total};
FIR (false identification ratio) = N_{false}/N_{total};
ER (error ratio) = $N_{false}/(N_{true}+N_{false})$;
N_{true}: number of sequences assigned to correct taxonomy. N_{false}: number of sequences assigned to false taxonomy. N_{total}: total number of sequences [1]

Table B4 Genera identified and associated relative abundance

Genera	Average relative abundance	Std. deviation	Max. relative abundance	Persistence
Penicillium	88.89%	6.37%	97.55%	100.00%
Aspergillus	0.71%	1.13%	6.15%	90.00%
Peniophora	0.29%	0.62%	3.03%	56.67%
Cladosporium	0.16%	0.28%	1.01%	50.00%
Rhodosporidium	0.18%	0.30%	1.48%	50.00%
Aureobasidium	0.25%	0.90%	4.95%	43.33%
Fusarium	0.14%	0.34%	1.57%	40.00%
Cantharellus	0.46%	0.93%	3.80%	36.67%
Trichosporon	0.09%	0.20%	0.90%	36.67%
Cryptococcus	0.07%	0.17%	0.75%	36.67%
Candida	0.15%	0.37%	1.59%	33.33%
Trametes	0.13%	0.34%	1.50%	33.33%
Pichia	0.87%	3.62%	19.78%	30.00%
Gymnopus	0.02%	0.07%	0.38%	26.67%
Acremonium	0.09%	0.27%	1.38%	26.67%
Wallemia	0.03%	0.06%	0.19%	26.67%
Rhizopogon	0.01%	0.04%	0.16%	20.00%
Basidiobolus	0.05%	0.15%	0.76%	20.00%
Stereum	0.04%	0.13%	0.63%	16.67%
Chaetomium	0.06%	0.19%	0.95%	16.67%
Eutypella	0.05%	0.12%	0.46%	16.67%
Mycoleptodiscus	0.01%	0.01%	0.06%	16.67%
Rhodotorula	0.02%	0.04%	0.18%	16.67%
Phanerochaete	0.03%	0.16%	0.89%	16.67%
Hyphodontia	0.05%	0.14%	0.56%	16.67%
Agaricus	0.01%	0.04%	0.16%	16.67%
Nigrospora	0.04%	0.09%	0.37%	16.67%
Inocybe	0.01%	0.03%	0.16%	16.67%

Table B4 (Continued)

Tricholoma	0.01%	0.05%	0.25%	16.67%
Alternaria	0.04%	0.14%	0.76%	16.67%
Glomus	0.01%	0.03%	0.16%	16.67%
Leptosphaerulina	0.03%	0.10%	0.44%	16.67%
Hypocrea	0.04%	0.18%	0.99%	13.33%
Amanita	0.00%	0.02%	0.08%	13.33%
Devriesia	0.11%	0.45%	2.31%	13.33%
Teratosphaeria	0.11%	0.43%	2.22%	13.33%
Toxicocladosporium	0.05%	0.13%	0.48%	13.33%
Sclerotium	0.02%	0.07%	0.38%	13.33%
Paecilomyces	0.04%	0.14%	0.77%	13.33%
Phoma	0.05%	0.16%	0.73%	13.33%
Phialosimplex	0.00%	0.01%	0.05%	13.33%
Polyporus	0.00%	0.01%	0.03%	13.33%
Eremascus	0.07%	0.30%	1.62%	13.33%
Epicoccum	0.03%	0.12%	0.63%	10.00%
Ceriporia	0.03%	0.12%	0.63%	10.00%
Sporobolomyces	0.03%	0.12%	0.63%	10.00%
Taphrina	0.00%	0.01%	0.08%	10.00%
Rhizoctonia	0.00%	0.02%	0.12%	10.00%
Eurotium	0.00%	0.01%	0.03%	10.00%
Phlebia	0.00%	0.02%	0.07%	10.00%
Olpidium	0.01%	0.03%	0.16%	10.00%
Oudemansiella	0.01%	0.04%	0.17%	10.00%
Heterobasidion	0.01%	0.06%	0.35%	10.00%
Davidiella	0.00%	0.02%	0.09%	10.00%
Lasiodiplodia	0.03%	0.16%	0.89%	10.00%
Xylaria	0.01%	0.06%	0.27%	10.00%
Flavodon	0.01%	0.04%	0.15%	10.00%
Cochliobolus	0.02%	0.09%	0.46%	10.00%
Apiosporina	0.00%	0.01%	0.06%	10.00%
Amorosia	0.00%	0.01%	0.02%	6.67%
Chaetocalathus	0.00%	0.01%	0.05%	6.67%
Waitea	0.01%	0.05%	0.25%	6.67%
Chromelosporium	0.00%	0.02%	0.08%	6.67%
Magnaporthe	0.08%	0.42%	2.31%	6.67%
Sarocladium	0.02%	0.07%	0.37%	6.67%
Falcocladium	0.00%	0.01%	0.02%	6.67%
Phomopsis	0.01%	0.04%	0.20%	6.67%
Daldinia	0.01%	0.06%	0.35%	6.67%
Caloplaca	0.01%	0.04%	0.22%	6.67%
Penidiella	0.00%	0.01%	0.04%	6.67%
Fuscoporia	0.01%	0.04%	0.19%	6.67%
Choanephora	0.01%	0.03%	0.13%	6.67%

Table B4 (Continued)

Rozella	0.01%	0.05%	0.27%	6.67%
Massarina	0.00%	0.02%	0.09%	6.67%
Dermocybe	0.00%	0.02%	0.08%	6.67%
Marasmius	0.00%	0.01%	0.06%	6.67%
Cystofilobasidium	0.01%	0.07%	0.38%	6.67%
Cladonia	0.00%	0.01%	0.07%	6.67%
Trichaptum	0.01%	0.03%	0.14%	6.67%
Scleroderma	0.00%	0.01%	0.06%	6.67%
Laetiporus	0.01%	0.06%	0.31%	6.67%
Oliveonia	0.02%	0.09%	0.51%	6.67%
Phialocephala	0.00%	0.01%	0.07%	6.67%
Dendrophora	0.04%	0.19%	1.04%	6.67%
Ceratocystis	0.01%	0.06%	0.32%	6.67%
Ambispora	0.00%	0.01%	0.03%	6.67%
Antrodiella	0.00%	0.01%	0.05%	6.67%
Phlebiopsis	0.00%	0.01%	0.08%	6.67%
Gibberella	0.00%	0.02%	0.07%	6.67%
Fomitopsis	0.01%	0.02%	0.11%	6.67%
Tubeufia	0.01%	0.03%	0.15%	6.67%
Cephaliophora	0.00%	0.01%	0.04%	6.67%
Entoloma	0.00%	0.01%	0.03%	6.67%
Bensingtonia	0.00%	0.01%	0.05%	6.67%
Coniothyrium	0.00%	0.02%	0.08%	6.67%
Hypochnicium	0.00%	0.01%	0.06%	6.67%
Exophiala	0.01%	0.04%	0.15%	6.67%
Diaporthe	0.01%	0.04%	0.18%	6.67%
Gongronella	0.02%	0.11%	0.63%	3.33%
Leohumicola	0.01%	0.05%	0.25%	3.33%
Schizophyllum	0.00%	0.02%	0.12%	3.33%
Dothidea	0.00%	0.02%	0.09%	3.33%
Nakaseomyces	0.00%	0.00%	0.02%	3.33%
Crinipellis	0.00%	0.00%	0.02%	3.33%
Hypoxylon	0.00%	0.01%	0.08%	3.33%
Aplosporella	0.00%	0.02%	0.12%	3.33%
Passalora	0.00%	0.00%	0.02%	3.33%
Paraphoma	0.03%	0.17%	0.95%	3.33%
Absconditella	0.02%	0.09%	0.48%	3.33%
Mycosphaerella	0.00%	0.02%	0.13%	3.33%
Agrocybe	0.00%	0.02%	0.13%	3.33%
Cantharellula	0.00%	0.02%	0.13%	3.33%
Phaeobotryosphaeria	0.00%	0.01%	0.06%	3.33%
Schizangiella	0.00%	0.00%	0.02%	3.33%
Ophiocordyceps	0.00%	0.00%	0.02%	3.33%
Hygrophorus	0.00%	0.00%	0.02%	3.33%

Table B4 (Continued)

Arthroderma	0.00%	0.00%	0.02%	3.33%
Cercospora	0.00%	0.01%	0.05%	3.33%
Ophiostoma	0.00%	0.00%	0.02%	3.33%
Hyphodermella	0.00%	0.02%	0.09%	3.33%
Pandora	0.00%	0.00%	0.02%	3.33%
Septoria	0.00%	0.00%	0.02%	3.33%
Auricularia	0.00%	0.01%	0.04%	3.33%
Kazachstania	0.00%	0.01%	0.04%	3.33%
Hormonema	0.00%	0.00%	0.02%	3.33%
Myrmecridium	0.01%	0.08%	0.43%	3.33%
Fomitiporia	0.00%	0.00%	0.02%	3.33%
Pleurotus	0.01%	0.03%	0.16%	3.33%
Serpula	0.00%	0.00%	0.02%	3.33%
Filobasidium	0.01%	0.05%	0.29%	3.33%
Calicium	0.00%	0.02%	0.13%	3.33%
Neurospora	0.02%	0.09%	0.49%	3.33%
Dendryphiella	0.00%	0.01%	0.03%	3.33%
Pyrenochaeta	0.00%	0.01%	0.06%	3.33%
Amylosporus	0.00%	0.01%	0.03%	3.33%
Phlebiella	0.00%	0.00%	0.03%	3.33%
Ganoderma	0.00%	0.00%	0.03%	3.33%
Phaeosphaeria	0.00%	0.02%	0.14%	3.33%
Hericium	0.01%	0.03%	0.19%	3.33%
Hyphoderma	0.00%	0.02%	0.14%	3.33%
Sporidiobolus	0.02%	0.12%	0.68%	3.33%
Skeletocutis	0.00%	0.01%	0.05%	3.33%
Artomyces	0.00%	0.01%	0.08%	3.33%
Ramichloridium	0.00%	0.01%	0.05%	3.33%
Cylindrobasidium	0.00%	0.00%	0.03%	3.33%
Rachicladosporium	0.00%	0.02%	0.14%	3.33%
Pterula	0.00%	0.02%	0.14%	3.33%
Nectria	0.00%	0.00%	0.03%	3.33%
Noosia	0.00%	0.02%	0.14%	3.33%
Lagarobasidium	0.00%	0.00%	0.03%	3.33%
Physisporinus	0.00%	0.01%	0.05%	3.33%
Rhizomucor	0.00%	0.00%	0.03%	3.33%
Dissoconium	0.01%	0.03%	0.16%	3.33%
Wickerhamomyces	0.00%	0.02%	0.12%	3.33%
Porpomyces	0.00%	0.00%	0.01%	3.33%
Lycoperdon	0.00%	0.00%	0.01%	3.33%
Trichoderma	0.00%	0.00%	0.01%	3.33%
Inonotus	0.00%	0.00%	0.00%	3.33%
Gigaspora	0.00%	0.00%	0.01%	3.33%
Preussia	0.01%	0.07%	0.41%	3.33%

Table B4 (Continued)

Trapelia	0.01%	0.07%	0.36%	3.33%
Paradictyoarthrinium	0.00%	0.03%	0.14%	3.33%
Metacordyceps	0.00%	0.00%	0.01%	3.33%
Golovinomyces	0.00%	0.01%	0.03%	3.33%
Beauveria	0.00%	0.00%	0.01%	3.33%
Valsa	0.00%	0.00%	0.02%	3.33%
Capnobotryella	0.00%	0.00%	0.02%	3.33%
Coriolopsis	0.00%	0.00%	0.02%	3.33%
Lecidella	0.00%	0.00%	0.01%	3.33%
Flagelloscypha	0.00%	0.00%	0.00%	3.33%
Sphaerobolus	0.00%	0.00%	0.00%	3.33%
Blakeslea	0.00%	0.00%	0.02%	3.33%
Gloeoporus	0.00%	0.00%	0.02%	3.33%
Hamigera	0.00%	0.01%	0.05%	3.33%
Pithomyces	0.00%	0.01%	0.05%	3.33%
Armillaria	0.00%	0.00%	0.02%	3.33%
Podoscypha	0.00%	0.01%	0.07%	3.33%
Ascochyta	0.00%	0.03%	0.14%	3.33%
Peyronellaea	0.01%	0.03%	0.17%	3.33%
Ustilaginoidea	0.00%	0.01%	0.03%	3.33%
Myrothecium	0.00%	0.01%	0.05%	3.33%
Debaryomyces	0.00%	0.01%	0.04%	3.33%
Ramularia	0.00%	0.01%	0.04%	3.33%
Corynespora	0.01%	0.03%	0.17%	3.33%
Pseudolagarobasidium	0.00%	0.03%	0.15%	3.33%
Psathyrella	0.00%	0.01%	0.03%	3.33%
Phialophora	0.01%	0.06%	0.31%	3.33%
Diatrype	0.01%	0.03%	0.15%	3.33%
Pseudocercospora	0.01%	0.03%	0.15%	3.33%
Acarospora	0.01%	0.04%	0.23%	3.33%
Saccharomyces	0.00%	0.01%	0.03%	3.33%
Plenodomus	0.00%	0.02%	0.09%	3.33%
Rhinocladiella	0.01%	0.06%	0.32%	3.33%
Cystidiodontia	0.00%	0.01%	0.06%	3.33%
Paraleptosphaeria	0.00%	0.01%	0.06%	3.33%
Myxotrichum	0.01%	0.08%	0.44%	3.33%
Degelia	0.03%	0.14%	0.77%	3.33%
Podosordaria	0.00%	0.01%	0.06%	3.33%
Suillus	0.00%	0.00%	0.02%	3.33%
Neosetophoma	0.01%	0.03%	0.16%	3.33%
Pisolithus	0.00%	0.00%	0.02%	3.33%
Ambiguous	5.50%	4.72%	22.22%	100.00%

Table B5 Shannon's equitability of each pool shows the fungal community is unequally distributed (ideally even community has index value of 1 by definition)

	June, 2011	Sept, 2011	Oct, 2011	Nov, 2011	Dec, 2011	Jan, 2012	Feb, 2012	March, 2012
Pool 1	0.28	0.46	0.41	0.17	0.20	0.19	0.09	0.09
Pool 2	0.22	0.26	0.13	0.15	N/A	0.13	0.15	0.30
Pool 3	0.19	0.11	0.18	0.13	0.17	0.06	0.17	N/A
Pool 4	0.09	0.14	0.19	0.16	0.33	0.34	0.14	0.11

N/A: Pool 2 from December and Pool 3 from March were PCR negative and not included in sequencing

Table B6 Water quality parameters (data derived from [2])

Month	Date	pH	Free chlorine (mg/L)	Monochloramine (mg/L as Cl_2)
T = 0 (Baseline: right before monochloramine injection started)	9/26/2011	8.3	0.03	0.00
Month 1	10/24/2011	8.6	0.14	3.14
Month 2	11/22/2011	8.1	0.09	0.76
Month 3	12/19/2011	8.1	0.15	1.60
Month 4	1/17/2012	8.0	0.22	2.58
Month 5	2/21/2012	7.9	0.17	2.80
Month 6	3/19/2012	8.1	0.13	2.57

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 5

C.1 ITS1 sequences of tap water isolates of *Aspergillus* and *Penicillium*

Aspergillus tap water isolate

5’-

GCNNNNNTCNNCCCGTGNTACCTAACACTGTTGCTTCGGCGGGGAACCCCCTCNNN

NNNNNGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTCT

GAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGA

AGAACGCAGCA -3’

The *Aspergillus* tap water isolate developed granular dark green colonies with cream to dark red back view on potato dextrose agar; was further identified as *Aspergillus versicolor* based on previously described colony morphology features [1, 2].

Penicillium tap water isolate

5’-

TCCNCCCTTGTCTCTATACACCTGTTGCTTTGGCGGGCCCACCGGGGCCACCTGGTC

GCCGGGGGACATCTGTCCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGAACCCTGA

TGAAGATGGGCTGTCTGAGTACTATGAAAATTGTCAAAACTTTCAACAATGGATCTC

TTGGTTCCGGCATCGATGAAGAACGCAGCA -3’

The *Penicillium* tap water isolate developed olive green colonies with cream back view and arial shape hypae on potato dextrose agar; was further identified as *Pencillium purpurogenum* based on previously described colony morphology features [3].

C.2 WinBUGS code for delayed Chick-Watson model fitting

```
Model
{
 for (i in 1:N) {
  Y[i] ~ dnorm(mu[i], tau)
  mu[i] <- beta * (CT[i] - CTlag) * step(CT[i] - CTlag)
   }
#non_informative priors (variance sigma2 = 1/tau, CTobs is the CT value corresponding to 10%
of lowest Ln(N/N0))
  tau ~ dgamma(0.001, 0.001)
  beta ~ dnorm(0.0, 1.0E-6)
  CTlag ~ dunif(0, CTobs)
  sigma2 <- 1/tau
}
```

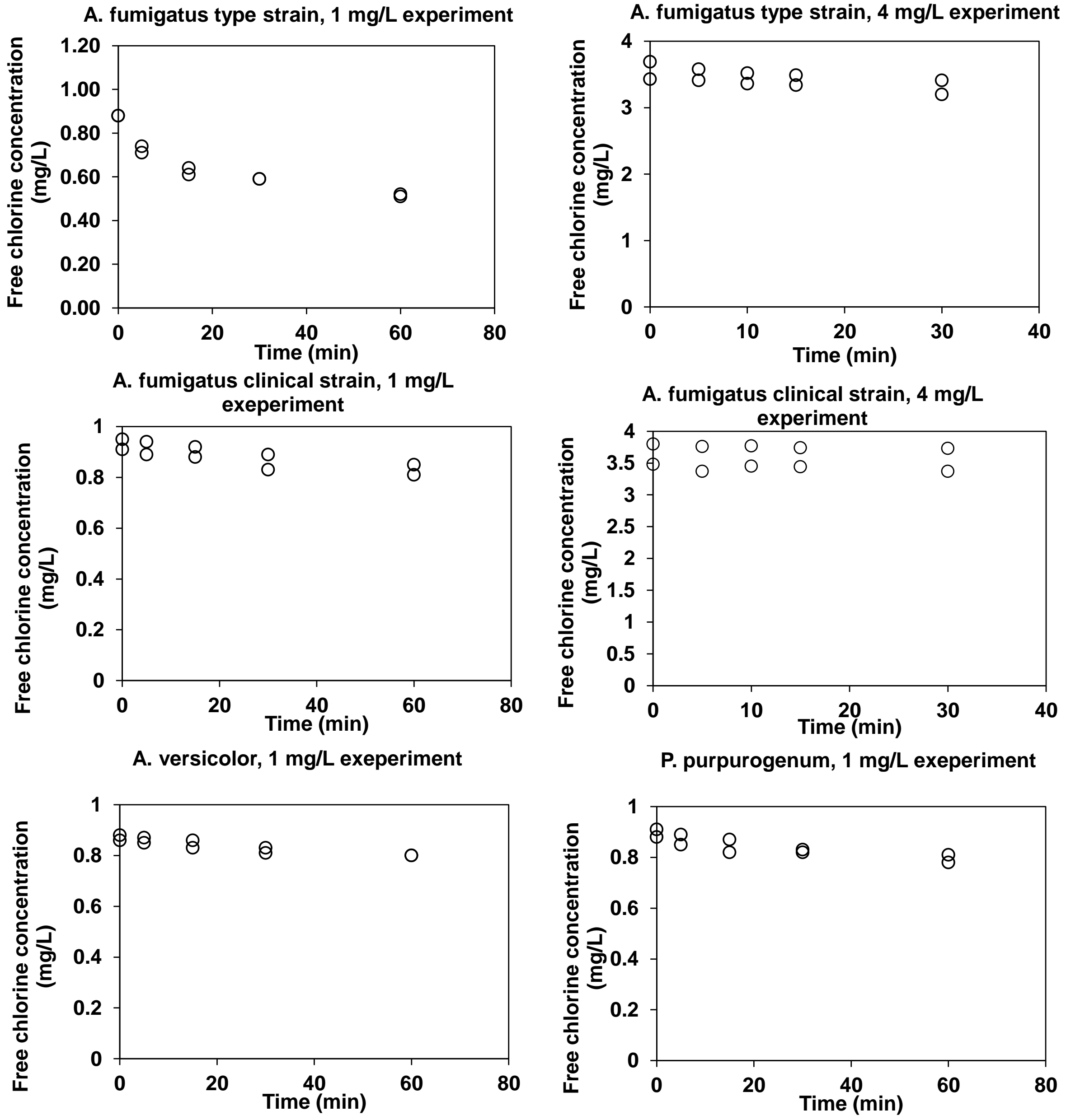

Figure C1 Measured free chlorine residual concentration (mg/L)

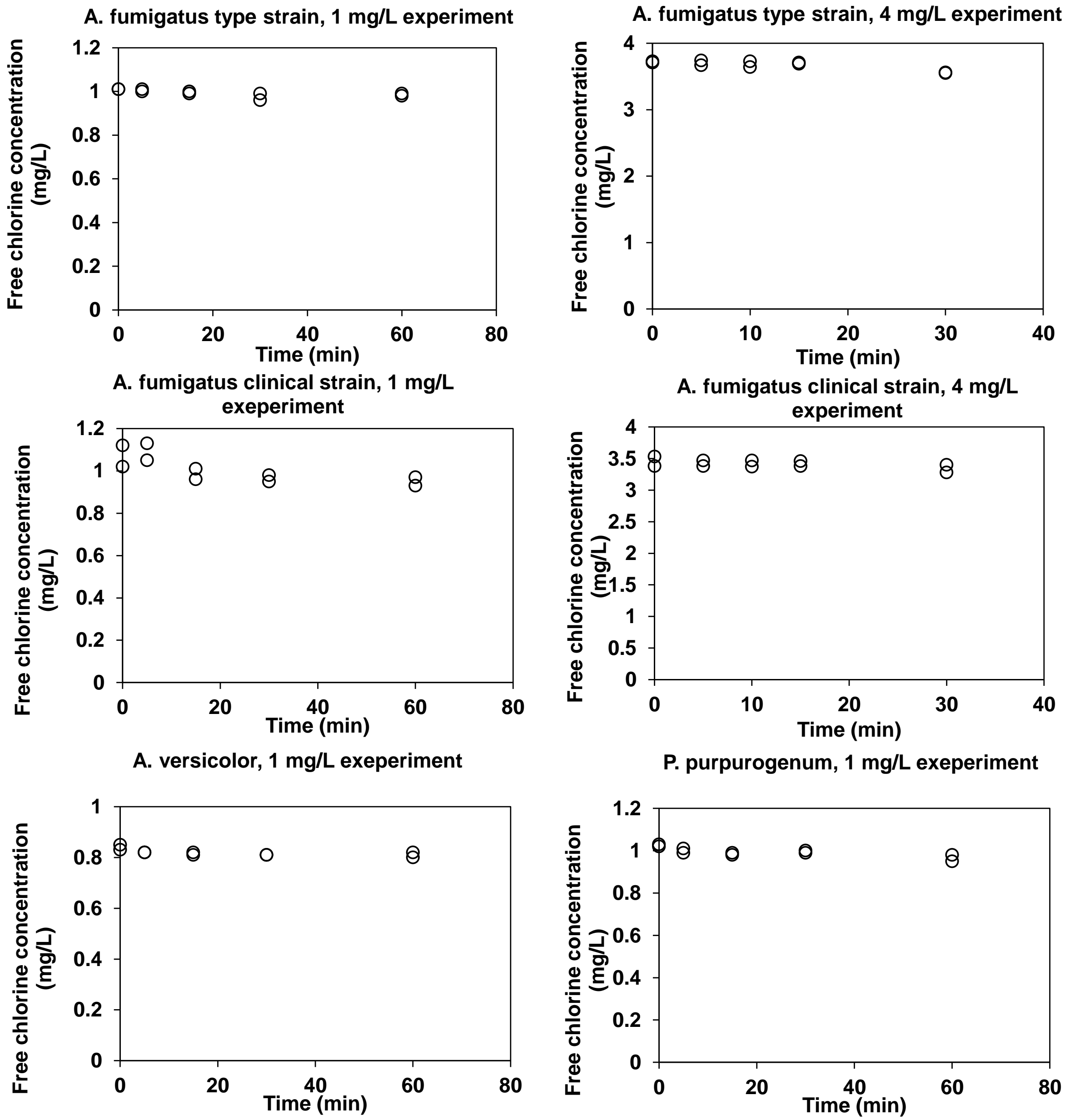

Figure C2 Measured monochloramine residual concentration (mg/L)

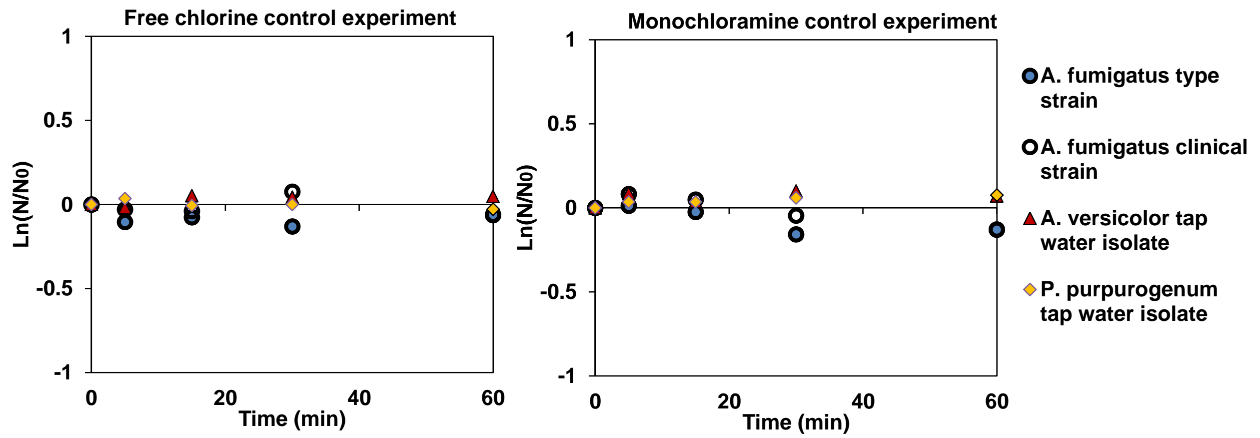

Figure C3 Measured mean $\mathrm{Ln}(N/N_0)$ for free chlorine and monochloramine control experiments, which no disinfectant was used

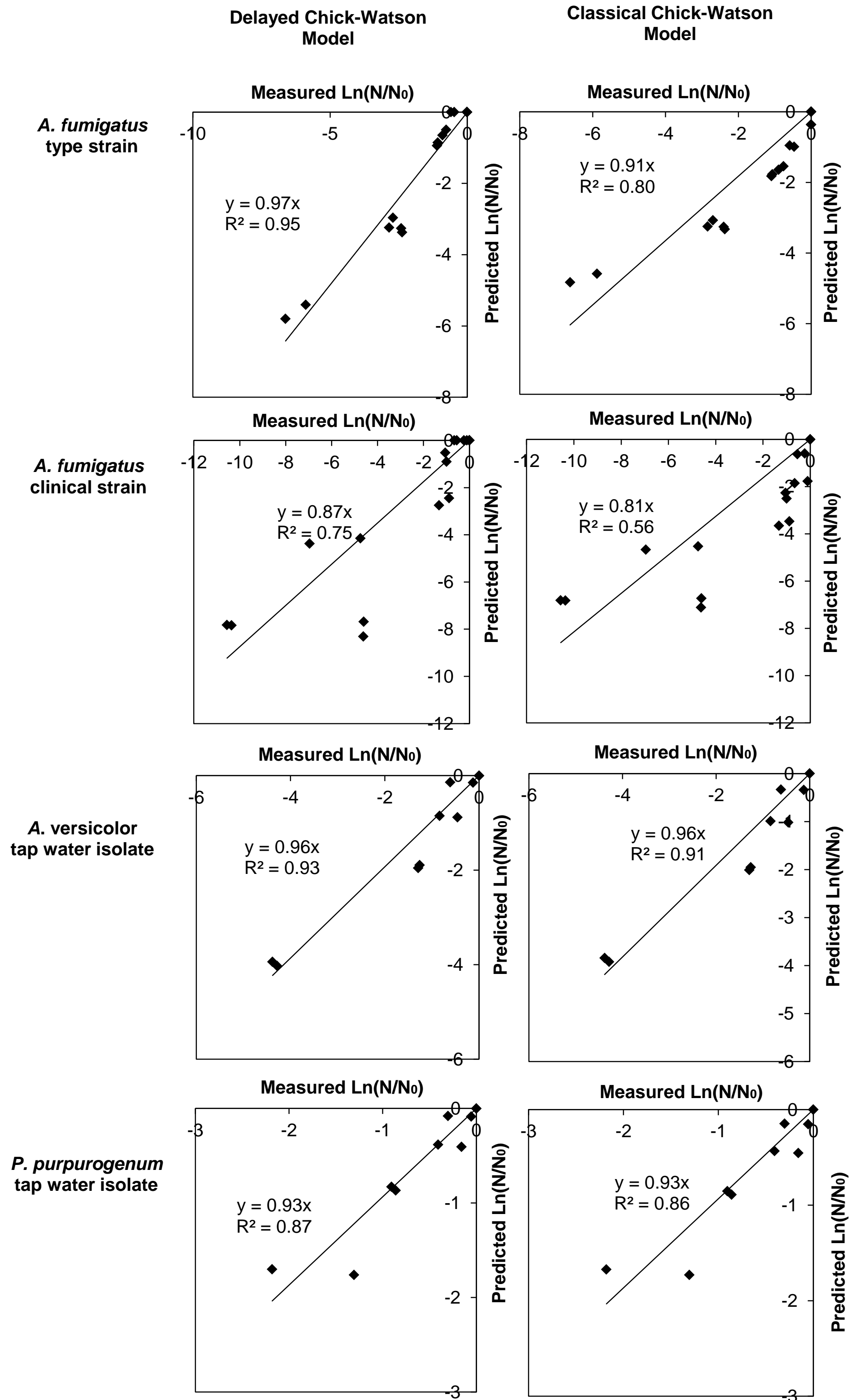

Figure C4 Comparison of model predicted $Ln(N/N_0)$ against measured $Ln(N/N_0)$ for both delayed Chick-Watson model and classical Chick-Watson model, free chlorine disinfection

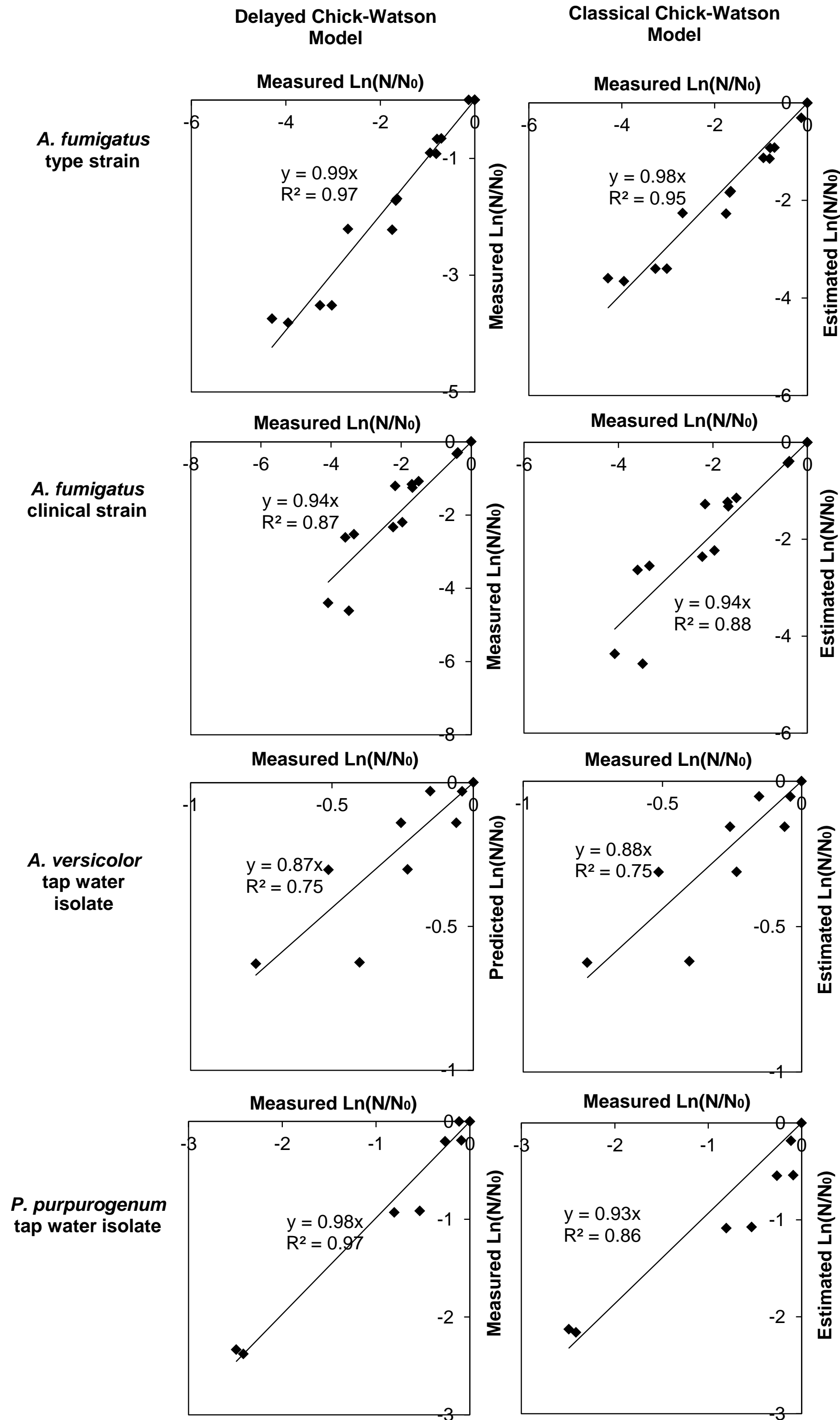

Figure C5 Comparison of model predicted $\mathrm{Ln}(N/N_0)$ against measured $\mathrm{Ln}(N/N_0)$ for both delayed Chick-Watson model and classical Chick-Watson model, monochloramine disinfection

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Appendix B

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Appendix C

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