Colonization of Granular Activated Carbon Media Filters By *Legionella* and Heterotrophic Bacterial Cells

by

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ABSTRACT

Granular activated carbon (GAC) filters are final polishing step in the drinking water treatment systems for removal of dissolved organic carbon fractions. Generally filters are colonized by bacterial communities and their activity reduces biodegradable solutes allowing partial regeneration of GAC’s adsorptive capacity. When the bacteria pass into the filtrate due to increased growth, microbiological quality of drinking water is compromised and regrowth in the distribution system occurs. Bacteria attached to carbon particles as biofilms or in conjugation with other bacteria were observed to be highly resistant to post filtration microbial mitigation techniques. Some of these bacteria were identified as pathogenic.

This study focuses on one such pathogen *Legionella pneumophila* which is resistant to environmental stressors and treatment conditions. It is also responsible for Legionnaires’ disease outbreak through drinking water thus attracting attention of regulatory agencies. The work assessed the attachment and colonization of *Legionella* and heterotrophic bacteria in lab scale GAC media column filters. Quantification of *Legionella* and HPC in the influent, effluent, column’s biofilms and on the GAC particles was performed over time using fluorescent microscopy and culture based techniques.

The results indicated gradual increase in the colonization of the GAC particles with HPC bacteria. Initially high number of *Legionella* cells were detected in the column effluent and were not detected on GAC suggesting low attachment of the cells to the particles potentially due to lack of any previous biofilms. With the initial colonization of the filter media by other bacteria the number of *Legionella* cells on the GAC particles and biofilms also increased. Presence of *Legionella* was
confirmed in all the samples collected from the columns spiked with *Legionella*.

Significant increase in the *Legionella* was observed in column's inner surface biofilm (0.25 logs up to 0.52 logs) and on GAC particles (0.42 logs up to 0.63 logs) after 2 months. *Legionella* and HPC attached to column’s biofilm were higher than that on GAC particles indicating the strong association with biofilms. The bacterial concentration slowly increased in the effluent. This may be due to column’s wall effect decreasing filter efficiency, possible exhaustion of GAC capacity over time and potential bacterial growth.
DEDICATION

This thesis is dedicated to my parents and everyone else who has helped me along the way including my fellow graduate students, friends and family as well as the faculty, staff, and committee members. If it was not for their timely support and guidance I would have never made it.

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LIST OF ABBREVIATIONS

1. GAC.................................................................Granular Activated Carbon
2. BAC.................................................................Biologically Activated Carbon
3. WTP.................................................................Water Treatment Plant
4. HPC.................................................................Heterotrophic Plate Count
5. U.S. EPA.....................................................United States Environment Protection Agency
6. WHO.............................................................World Health Organization
7. THM...............................................................Trihalomethanes
8. HAA...............................................................Halo Acetic acid
9. VBNC...........................................................Viable But Non-Culturabl
10. DI ..................................................................Distilled
11. PVC..............................................................Poly Vinyl Chloride
12. MDS...............................................................Model Distribution System
13. MCL.............................................................Maximum Contaminant Limit
14. SEM..............................................................Scanning Electron Microscopy
CHAPTER 1
INTRODUCTION

MOTIVATION FOR STUDY

Granular activated carbon (GAC) filters are very common in drinking water treatment plants since 1930s in the United States. The removal efficiency of organic matter from raw water has been improved by using GAC as a filtration media instead of sand in most conventional water treatment plants. GAC’s effectiveness is due to its irregular creviced, porous particle shape and ability to attract specific contaminants. It helps remove undesirable dissolved organic carbon (DOC) fractions, biodegradable organic substances, micropollutants, halogenated hydrocarbons, taste and odor compounds and other contaminants such as humic and fulvic acids which are naturally produced (16, 53). The GAC treatment process also reduces organic or precursor compounds that produce trihalomethanes and other disinfection by-products upon reaction with chlorine. Removal occurs via adsorption. Solutes are adsorbed on the adsorbent that is GAC (16).

Generally filters are subjected to bacterial colonization soon after it starts operating. Bacterial growth is an expected consequence due to the favorable environment provided by the filter. Colonization occurs as a result of the adsorptive properties of carbon which serves to enrich organic nutrients and oxygen for bacterial attachment and removes disinfectants harmful to these bacteria. Further, the porous structure of GAC acts as a protection from fluid shear forces. Laboratory experience indicates that operating an activated carbon filter is nearly impossible without bacterial growth. Such GAC filters are commonly known as Biologically Activated Carbon filters (BAC) (53).
Bacterial genera such as *Flavobacterium, Bacillus, Pseudomonas, Aeromonas* and others have been identified in the GAC filters. The distribution of these genera largely depends on the source water characteristics (12). It was initially observed that the growth of microorganisms was beneficial in the removal of organics. The indigenous bacterium which colonizes the filter and establishes biofilms consumes biodegradable organic matter and thus lowers the disinfectant demand and reduces the formation of disinfection byproducts. The biodegradable organics are broken down by the biofilms to biomass, carbon dioxide and waste products before these are adsorbed on the GAC. This prolongs the carbon bed life for removal of recalcitrant and non-biodegradable organics. These beneficial effects have led to a growing trend of intentionally incorporating the BAC filters into the treatment system. Despite the full scale extensive application of this technology very little is known about the indigenous and concomitant microbial communities that colonize the filter opportunistically (5).

Detailed investigations revealed problems associated with BAC filters. The major ones are the penetration of bacterium coated GAC particles through the treatment barriers and the sloughing or shearing of organisms from colonized filter beds into the effluent drinking water (12, 45). The occurrence subsequently in the filtrate due to increased growth and penetration compromises the microbiological quality of drinking water and invites consumer complaints (12). Some of these microbes were identified as pathogenic (29). Usually, the concentrations of microbes in the filtrate are easily regulated with post-filtration techniques like disinfection to regulatory standards. However, few microbes attached to carbon particles as biofilms or in conjugation with other bacteria were observed to be highly resistant to
disinfectants. These might have pathogenic relevance or accumulate as biomass in reservoirs and stagnant pipes leading to microbiological regrowth problems in the distribution system (29). Backwashing as a remedy to the problem failed and all the more exaggerated the problem by immobilizing the microbial colonies in the filters (22).

This study is focused on one such pathogen namely *Legionella pneumophila* which is highly resistant to environmental stressors like disinfection and its colonization of GAC filter media over time along with heterotrophic bacteria (1). It evaluates the potential of bacterial growth and colonization in the drinking water treatment system. The study quantifies the potential colonization and attachment of a specific pathogenic bacterium *Legionella* and other heterotrophs in the filter, and analyzes their presence in lab scale GAC media filter column effluent.

**STUDY OBJECTIVES**

The main objective of this study is to examine the potential growth and colonization by indicator and pathogenic bacteria on the GAC media filter using tap and settled source waters.

The specific objectives covered by the experimental chapter in the report are as follows:

1) To analyze potential growth and colonization of GAC media filter by heterotrophs.

2) To analyze potential growth and colonization of GAC media filter by *Legionella*. 
3) To quantify the heterotrophic bacteria and *Legionella* in the effluent of lab scale GAC media filter columns.

In each objective the primary finding will focus on the presence, attachment, potential growth, and colonization of GAC media filter with heterotrophic bacteria and *Legionella*. The following section will describe the general material and instrumentation employed in experimental analysis. Individual analysis procedures will be addressed in the experimental chapter under the Materials and Methods section.
CHAPTER 2

LITERATURE REVIEW

Safe, clean and healthy drinking water is basic necessity of life. Streams, lakes and rivers can be a source of drinking water or it can be directly collected and stored from rain. It can also be acquired by desalination of ocean water and melting of ice, or extracted from groundwater resources. Based on various sources different water quality issues have to be faced when utilizing these. With increasing population there has been over-exploitation of water resources. Surface water gets exposed to pollution by human wastes, microbes, nutrients such as nitrate and ammonium and spills of toxic chemicals (43).

World Health Organization (WHO) has set guidelines for drinking water quality all over the world. The guideline deals with the protection and improvement of water quality including selection of water resources, treatment methods, distribution methods and emergency measures (25). Specific guidelines are also available for acceptable concentrations of (a) bacteria, viruses, parasites (b) chemicals of health significance organic and inorganic constituents, pesticides, disinfection by-products (c) radioactive constituents (d) substances and parameters in drinking water that may give rise to complaints from consumers. The U.S. Environmental Protection Agency (USEPA) in the United States is responsible for establishing national drinking water regulations under the Safe Drinking Water Act (SDWA), originally enacted in 1974 and further reauthorized in 1986 and 1996 (21).

The connection between bacteria in drinking water and the outbreak of various diseases triggered the necessity of protecting resource and drinking water treatment. A conclusive chain of evidence was obtained in 1919 when the typhoid
fever outbreak in Germany resulted in 400 deaths and 4000 cases of typhoid fever due to contamination of drinking water by sanitary waste applied as fertilizer. As a result, areas which were sources for drinking water production were protected, and practices to decontaminate treated water to remove as many of the remaining bacteria as possible were established. A multiple barrier concept involving source water protection, optimization of water treatment processes and maintained distribution system ensures safe water for all (50).

High standards are set for quality and safety of water. The bacterial content should be very low and non-detectable for pathogenic microbes as per strict requirements for microbiological factors. With continuous evolution in treatment practices and discovery of new pathogenic microorganisms in the environment a broader attitude is needed towards the occurrence of potentially pathogenic bacteria, viruses and parasites. Therefore, regulations, guidelines and legislations by like the World Health Organization have become stricter and lowered the acceptable limit of occurrence of such microbes in drinking water to levels where these do not cause waterborne infections. These regulations are fulfilled at several stages like resource protection, careful treatment of raw water and maintaining a regular check on treatment processes. Evaluation of the behavior of pathogens in drinking water is also important factor to further revise treatment technologies, processes and regulations (25).

**RELEVANT REGULATIONS**

Drinking water regulations including the enforcement of regulatory standards, the required monitoring, application of specific treatment processes and
the submission of reports regarding compliances of these regulations by treatment facilities is determined by the U.S. EPA under the Safe Drinking Water Act of 1974.

**The Surface Water Treatment Rule (U.S. EPA, 1989a).** The rule requires maintaining disinfectant residual in the distribution system and was effective from December 31, 1990. A continuous monitoring is required for the presence of HPC or disinfectant residual in public water systems. Especially, if a system is avoiding filtration the disinfectant residual in the distribution system cannot be undetectable in more than 5% of the samples in a month for any 2 consecutive months that the system is running to serve the public. The number of samples is usually determined by the number of customers served by the system. HPC can be measured instead of a disinfectant residual. A HPC less than or equal to 500 CFU/mL is considered equivalent to detectable residuals of disinfectant. The rule goes along with the Total Coliform Rule (2).

**The Coliform Rule (U.S. EPA, 1989b).** The rule established a Maximum Contaminant Level Goal (MCLG) of zero for total coliforms in the distribution system and was effective December 31, 1990. A MCL of no more than 5% of the monthly samples is allowed to be coliform positive if 40 samples are analyzed per month. For systems less than 40 samples/month the limit drops to no more than one sample/month as coliform positive. As high HPC interferes with coliform detection the two monitoring needs to be done at the same location with 24 hours of the first one to validate the presence of coliform in the sample (3).

**The Information Collection Rule.** The rule required data collection and monitoring of microbial pathogens, fecal contamination indicators, disinfectant dose, and
disinfection byproducts such as THMs and HAAs. It was implemented by the USEPA in 1998. The main goal was to assess the potential health risks, make public health decisions and help structuring future regulations. The rule widely applied to public water systems serving a large number of people using surface water or water which was under direct influence of surface water for 18 months.

**FILTRATION**

Traditionally drinking water treatment involved chemical coagulation, sedimentation in clarifiers, sand/gravel media filtration, chlorine disinfection treatment and finally distribution to customers. A common practice since the inception of the water treatment plant for cleaning surface water for drinking purposes has been filtration. Filtration is the last barrier or step in a water treatment plant. It achieves removal of turbidity and microorganisms from water. Filters are the final obstacle for keeping the particles and protozoan cysts to release into the distribution system. Water passes through a pore structure formed by different types of bed materials in a filter. The bed material can be made of sand (sand filtration), a layer of diatomaceous earth or a combination of coarse anthracite coal overlying finer sand (dual or tri-media filtration) (9). The various processes involved during filtration for particle removal are by straining through the pores, by adsorption of the particles to the filter media, by sedimentation in the media pores, by coagulation traveling through the pores and by biological mechanisms as in slow sand filtration.

It was observed that removal efficiency of organic matter largely improved by using granular activated carbon (GAC) as the filtration media instead of sand in
most conventional treatment plants. GAC has a porous particle structure, creviced and an ability to bind specific contaminants (46).

**Granular activated carbon filtration.** GAC has been used successfully in the drinking water treatment settings for a long time. Due to its high organic removal efficiency several hundred municipalities used GAC in 1977 as a substitute for powdered activated carbon. In the United States, since 1960s GAC is generally used to remove taste and odor problems or volatile organic compounds (VOCs) from groundwater, and as a filtration media.

GAC plays a dual role. In addition to its adsorption properties it is also an effective filter medium. Apart from taste and odor compounds organic substances are also removed by GAC. GAC is created using carbon-based raw materials like peat, wood, coal etc. These are converted to char by carbonization and then oxidized to activate in order to create an internal pore structure. GAC is commonly made by treating hydrothermally different carbon based solids like coal of the types - bituminous, sub-bituminous or lignite. The starting material has a huge impact on the adsorption and filtration parameters of the finished product. Narrow fissures between the graphene planes are created by the pyrolysis step in the hydrothermal treatment. Further oxidation step facilitates the gasification of some of the graphene layers so as to create slightly wider spaces between the layers.

Presently, GAC helps in removal of dissolved and suspended particles in solution which eventually results in the removal of color, taste and odor causing compounds, certain synthetic organic compounds (SOCs), disinfection by-product (DBP) precursors, natural organic matter (NOM) and heavy metals. GAC adsorption
is attributed to mass transfer processes involving surface chemistry of interacting particles. A particle follows few steps in the adsorption process (47):

1. **Bulk solution transport**: where the adsorbate is transported towards the adsorbent from bulk fluid via diffusion or mixing.

2. **External film transport resistance**: molecular diffusion occurs to transport the adsorbate through stationary boundary layer of water surrounding adsorbent.

3. **Internal pore transport**: Adsorbate is transported to adsorbents available pore space to ensure adsorption.

4. **Adsorption**: Bonds are created between adsorbate and adsorbent sometimes also via chemical reactions, after adsorbate occupies available adsorption site.

Granular activated carbon has both physical and chemical properties that make it a good adsorbent. The porosity of the GAC, provides a high surface area, and thus makes it a good absorbent. A major limitation to GAC filtration is organic matter saturation which is when all of its available adsorption sites are non-selectively bound to organic matter or microorganisms. Once it’s saturated it is called exhausted or spent GAC. Spent GAC lets organic matter to pass through the filter and cause water quality issues downstream like bacterial growth in the
distribution system. Depending on nutrient loading GAC has a life of almost 6 to 12 months.

Spent GAC can be regenerated or replaced. Regeneration is the process of removal of adsorbents which adsorbed on the GAC surface and is more cost effective of the two. This reactivation is an added cost to the use of GAC as it allows for change in mass and pore size distribution. In conventional treatment plant, GAC is regenerated up to much of its original capacity. GAC can be regenerated many times before its performance starts to deteriorate and reaches a point where it is no longer economically beneficial to regenerate it further.

**Biologically Activated Carbon Filter.** Biologically activated carbon (BAC) filtration is a water treatment biotechnology which has emerged recently and is capable of overcoming several limitations associated with conventional and advanced treatment processes. The BAC filters emerge from the basic GAC filter media. It was observed that filters in which GAC was not replaced or regenerated on a regular basis evolved into what is known as biofilters. After exhaustion of the GAC media the rough porous surfaces of the GAC particles become amenable to microbial colonization which grows into significant biomass or “biofilm” (51). This biofilm then processes and degrades a fraction of bound waterborne nutrients and contaminants in the GAC pores. Biofilters are filters where most of the dissolved organic carbon (DOC) removal is due to biodegradation instead of adsorption (54). Biodegradation occurs due to the colonization of GAC particles by indigenous microbial communities hence these filters are also named as biologically activated carbon (BAC) filters. The transition is time dependent and changes the system performance considerably. This
colonization of the GAC filter is thought to result from the adsorption by carbon which adsorbs nutrients and oxygen at the same time removing disinfectant compounds harmful for bacterial growth. Apart from this, the porous surface of the GAC particles provides for a protective environment from fluid shear forces and the functional groups on the particles enhances bacterial attachment.

BAC process reduces the dependence of water treatment processes over chemical disinfection along with decreasing the frequency of filter backwashing. This extends the service life of the conventional GAC filter. BAC targets the biodegradable organic carbon fraction and thus removes less DOC overall than usual GAC filters. When a GAC/BAC filter is placed just after an oxidation step it enhances the biodegradable fraction of DOC by oxidizing DOC into low molecular weight oxygen containing organic molecules (53). The removal of the biodegradable fractions also reduces the formation of undesirable disinfection by-products and the regrowth of bacteria in the water distribution system. In addition, BAC eliminates the need for coagulation that was traditionally used to support water filtration process (46). A number of genera are identified for example *Bacillus, Pseudomonas, Flavobacterium, Aeromonas, Chromobacterium, Alcaligenes* and others. These genera vary significantly in the effluent waters based on the different characteristics of water, modes of treatment plant operation and varying bacterial enumeration and identification methodologies (48).

Utrilla et al. investigated the adsorption of *Escherichia coli* on different activated carbons. These carbon samples were characterized based on their surface area, pore size distribution, elemental analysis, mineral matter analysis and pH of the point zero charge. It was observed that the adsorption capacity of the activated
carbon samples increased with their hydrophobicity and macropore volume (44). Moreover in the presence of cations like Fe3+, Ca2+ and Mg2+ the bacterial cells showed varied adsorption ranging from 25% up to 88%. This was explained due to the reduction in electrostatic free energy and the increase in cell surface hydrophobicity. Consequently, porosity of carbon decreased and negative charge density increased due to adsorption of bacteria on activated carbon.

Composition of microbial community colonizing GAC to form BAC filter is very limited till date. The composition gives important information about the biodegradation capacity of the BAC filter (34). Many environmental factors such as dissolved oxygen, nutrients and organic matter can affect the composition of microbes forming biofilm on GAC particles (31). Nutrients are seen to gradually decrease as they are consumed by the organisms along the filter depth. There is also little knowledge about the spatial heterogeneity of microbial structures in drinking water biofilter (22).

Velten et al. followed the biofilm formation and development in a GAC filter on pilot scale for the first six months of its operation. They used adenosine triphosphate analysis to measure attached biomass at four different depths in the filter. They observed that the biomass increased rapidly during the first 90 days of operation at all depths in the filter and maintained a steady state afterwards. During the start-up and steady state vertical gradients of biomass density and growth rates were observed. An increased growth in the filter as well as occurrence of suspended bacteria in the effluent was detected due to increase in influent assimilable organic carbon concentration (53).
Boon et al. also studied microbial community dynamics at different depths of an undisturbed pilot scale GAC filter treating ozonated lake water during the start-up and maturation phase over a period of 6 months. They tried to correlate filter performance to microbial activity and stated that microbial gradients developing in the filter were important. The results of the study showed that the microbial community dynamics and species richness increased along the GAC bed depth, the community organization of the GAC stabilized and evened out with increasing reactor operation time at all the depths and that the specific community composition in the filter determined the DOC removal efficiencies along the bed and caused nutrient stratification. This information can help plan management of different microbial communities in drinking water plants to obtain a desired performance from the filter (10).

BAC filters are beneficial in removing organic matter or biodegradable matter from drinking water which is the prime contributor to heterotrophic regrowth in the distribution system regardless of the presence of high disinfectant residuals (28). Hence, it helps in biological stability of the water. This way environmental engineers and ecologists use this information to correlate microbial community structure and dynamics with system performance and change the system to get the benefits of the BAC process. Therefore, it depicted that higher community biodiversity lead to increased ecosystem stability as the ecosystem is protected through functional redundancy if many species co-exist. Optimization of BAC reactor performance can be made much easier based on a straightforward analysis of the natural microbial communities, scrutiny of community dynamics and functional organization.
While bacteria have a beneficial and significant recognized function in GAC filter still very little is known about the microbial community distribution and dynamics in BAC filters. A lot of this information is based on cultivation depended plating method. Although the function of these organisms in GAC filters has been found to be useful in removal of disinfection by-products and compounds causing taste and odor, GAC treatment might rather increase the number of bacteria entering the distribution system. Only recently the potential harmful impacts of these attached organisms in the distribution system have been studied (12). Wilcox et al. investigated the microbial dynamics associated with granular activated carbon in a pilot water treatment plant over a period of 16 months. They observed a gradual growth in the microbial count in the effluent water indicating microbial growth on the GAC particles. They examined the GAC particles and the interstitial waters and found organisms like *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, and *Pseudomonas*. Total Coliform bacteria were also seen to increase in small counts mostly during later months of the study (55).

Camper et al. examined various bacteriological effects of GAC in treated water. They studied the release of coliforms and HPC bacteria, the impact of EBCT on the release of particles and bacteria, and the bacteriological activity associated with GAC particles. Potential problems were seen to arise if GAC particles coated with bacterium penetrates the treatment barriers or if the colonized organisms are sheared from the filter beds and enter the effluent drinking water (12). LeChavellier et al. presented evidence of the occurrence of carbon particles in the finished effluent water of drinking water treatment plants using GAC as the final treatment process.
Particles were collected using gauze filters through the entire filter run as well as before and after backwashing. They found the fines were released throughout the filter cycle with no peaks in the breakthrough during the start or end of the cycle (29). They followed up with another study using homogenization procedure indicating that nearly 42% of the water samples had HPC bacteria attached to carbon particles. The coliforms attached to these particles were mostly of the fecal biotype. Scanning electron micrographs of these carbon fines from treated water showed micro-colonies of bacteria on the particle surfaces showing that these fines can be an important reason of regrowth in the distribution system (12).

Further experiments were conducted by the group to find out whether the bacteria attached to these carbon particles in effluent can survive disinfection better than non-sorbed bacterial cells. The bacteria were grown on the carbon particles and then disinfected with 2.0 mg of chlorine per liter for an hour (leaving 1.4 to 1.6 mg of free chlorine residual after 1 hr). These attached bacteria were found resistant to disinfection by chlorination when attached to carbon particles as no significant decrease in the viable counts was observed. Scanning electron microscope indicated that GAC is usually colonized by bacteria and pathogens in its cracks and is coated by a slime extracellular layer this mechanism suggests a possible explanation by which they penetrate disinfection and treatment barriers and enter drinking water supplies (29).

Similar experiments were extended to three water borne enteric pathogens such as *Salmonella typhimurium, Yersinia enterocolitica, and Shigella sonnei* which were analyzed for their ability to colonize GAC in pure cultures and in the presence of autochthonous river water organisms. All three readily colonized virgin GAC but
the colonization was limited by the presence of autochthonous microbial community. It was observed that in the presence of native aquatic bacteria the growth and colonization of GAC by pathogens declined at different rates depending on when the indigenous bacteria were introduced. This demonstrates the importance of indigenous bacteria in controlling human pathogenic bacteria on GAC (13).

Consequently, they checked the efficiency of disinfection on GAC fines colonized by these pathogens. For this they set up experiments using two attachment procedures – first letting them grow on the carbon naturally and the second to attach these from above manually. A control of attached and unattached cells was chlorinated with the same 2.0 mg of free chlorine per ml for 1 h as in their earlier experiments. They observed that unattached cells survived no more after 5 min of disinfectant contact time while attached cells experienced a small decrease in viability in the presence of chlorine as well as a varying amount of injury happened to the cells. The cells survived disinfection even better when attached to the GAC surface. Coliforms, pathogens and heterotrophic plate count bacteria were all reported to survive high chlorine doses for long contact time periods without any loss in the viability (29).

The study by LeChavellier et al. thus demonstrates that suspended particulates especially GAC have a harmful impact on the quality of drinking water. These particles might also be responsible for regrowth problems in the distribution system if these coliform associate with biofilms or plankton inside the system. They also showed a decreased in disinfection efficiency of approximately 90% if the turbidity of drinking water was increased from 1 to 10 nephelometric turbidity units (12, 29, 13, 30).
Parker and Darby proved using various blending and homogenization techniques for extracting particle associated coliform that significantly higher coliform is present in secondary effluents than is indicated by the standard enumeration procedure (37). Pernitsky et al. repeated similar kind of experiments and developed methods at bench scale to identify and enumerate the bacteria attached to GAC which has survived disinfection. This method helped to determine whether a significant amount of bacteria was crossing the treatment and disinfection barrier via attaching to fine GAC particles. They used a physical/chemical desorption technique to assess the impacts of colonized GAC fines being released in the effluent water of a BAC using treatment plant. Desorption technique was used on the laboratory colonized GAC fines which consisted of homogenization at 16,000 rpm at 4°C in a solution containing 0.01M Tris buffer at pH 7. After homogenization HPC bacteria were enumerated using membrane filtration. For a significant number of attached bacterial counts on the GAC fines the increase in the bacterial recovery from homogenized sample over that of non-homogenized sample was observed. A two fold or greater increase indicated significant attached bacteria on the fines. When suspensions with high concentration of GAC fines (1-100mg/l) were disinfected the attached bacteria were found to be more resistant to chlorination than unattached bacteria (40).

The harmful effects of attached bacteria in GAC effluent water can be controlled if –

1) There is increased awareness of health authorities, plant operators and engineers of the potential microbiological problems associated with using activated carbon in the treatment system.
2) Turbidity increases and carbon breakthrough are monitored timely and acted upon accordingly.

3) Free chlorine residual is maintained in all parts of the distribution system to curb bacterial growth on carbon particles in drinking water as much as possible.

4) A continuous flushing program is established to remove sediments and particles from distribution system.

Apart from this further comprehensive study and research is still required to assess the amount of carbon penetration through GAC filters, to fully understand the microbiological health effects associated with the use of BAC filters and complete characterization of microbial community growing on these carbon particles.

Several studies concluded that almost 95% of all microbial cells present in drinking water distribution systems exist as biofilms and rest 5% occur in the water phase (25, 6). Most of these biofilms consist of autochthonous aquatic microflora without any relevance to human health but these can opportunistically be inhabited by some pathogens. These pathogens can be of potential health hazard to humans when exposed. Many such pathogens get protection from environmental and external stresses when they are integrated in the biofilms. A lot of them have shown resistance to action of disinfectants and multiply in the biofilm. These pathogens become human health threats and contaminate drinking water when they are released from these biofilms due to physical shearing, shredding and disturbance which leads to subsequent detachment (24). Several such pathogens which can integrate into biofilms have been reported such as *Pseudomonas aeruginosa* and *Legionella pneumophila*. *Legionella* has been shown to be associated with biofilm in
warm water plumbing systems and is seen to be multiplying in hosts like free-living protozoa (32).

**LEGIONELLA SPECIES**

The *Legionella* bacterium is a public heath interest organism as it has the ability to infect humans and is almost present ubiquitously in the different environment (52). The *Legionella* bacterium is the causative agent of pneumonia and is therefore also called *Legionella pneumophila* (11). It was discovered following the 1976 pneumonia outbreak in Philadelphia and is an opportunistic pathogen. The word pneumophila means “lung-loving”. The disease caused by its infection is termed the Legionnaires’ disease. The bacteria can also cause Pontiac fever which has symptoms similar to influenza. The number of cases in the United States reported rose from 3.5 per million populations in 1984 to 6.3 per million in 1994 and then started to decline to 4.7 in 1996 (23).

*Legionella* genus consists of 42 species out of which eighteen have been linked to patients with pneumonia. The majority of human infections are due to *L. pneumophila*. These are gram negative rod shaped small bacteria which are un-encapsulated and non-spore forming. They are aerobic, microaerophilic and derive their energy and carbon sources through catabolism of amino acids. They primarily occur in aquatic environments although ubiquitous in nature. It has been observed in freshwater, marine waters, groundwater as well as potable water supplies. They survive in different conditions in water like in temperature range of 0-63°C, pH ranges of 5.0-9.2 and dissolved oxygen of concentration 0.2-15 ppm in water (35).
Swab samples are the best way to analyze the presence of *Legionella* in water. Samples are further concentrated by filtration and pretreated with acid buffer to enhance *Legionella* recovery (19). Acid treatment isolated *Legionella* as these are resistant to it compared to other bacteria. These samples then are plated on proper media which contains complex nutrients like high iron content. Commonly selective buffered charcoal yeast extract (BCYE) media buffered to pH 6.9 supplemented with ketoglutarate, L-cysteine, iron salts is used to culture *Legionella*. This can be further supplemented with antibiotics which help suppress the growth of other microorganisms like cefamandole, vancomycin, polymyxin B and anisomycin. A rapid test for *Legionella* detection used in the Direct Immunofluorescence Assay (DFA) (20).

*Legionella* proliferates by depending on other microorganisms through symbiotic relationships. They have been seen to multiply in water which already has presence of other microorganisms compared to sterile water (49). Thirteen species of amoebae and two species of protozoa are known to be infected by *Legionella*. These hosts provide protection to the bacteria from severe environmental conditions. Hence, it has been studied that these are able to survive in higher temperature range, dry conditions if encapsulated in cysts and show resistance to water treatment with disinfectants like chlorine. Cooper and Hanlon developed *Legionella pneumophila* biofilms on copper and stainless steel, commonly used in distribution systems. They conducted experiments where the biofilms were exposed to chlorine disinfection for three days and three month period. The bacterium was seen to survive in low numbers for 28 days in the presence of chlorine. Further, they grew biofilms for 3 days (immature biofilm), a month and 2 months (mature biofilms) respectively on
stainless steel and copper sections. These were exposed to 50 mg/l chlorine for 1h. There were no recoverable colonies immediately after exposure but the colonies started appearing over the following days in low numbers. The biofilms continued to grow and showed an increase in colonies. The experiments indicated high levels of resistance to chlorination by the *Legionella* biofilms. This resistance to disinfection has consequences in the form of disease transmittance through distribution system and problems in various treatment procedures (15).

*Legionella* has been observed growing symbiotically with aquatic bacteria attached to the surface of biofilms. Biofilms serve as a source of nutrients for growth and protection from adverse environmental conditions like treatment with disinfectants. Biofilms are common in drinking water distribution systems as well as BAC filters they act as suitable habitat for *Legionella* to grow in drinking water which can lead to problems when human exposure occurs. *Legionella* was reported in plumbing fixtures in potable water systems by British investigators in 1980. It has been detected in all segments of community water supplies including water treatment facilities. Though most of the disease outbreaks have been linked to heat exchange units in hospitals, hotels, industries etc. but with increasing awareness of its survival the attention has slowly shifted to potable distribution systems as it can be an important source of human infection (27).

*Legionella* have been seen to survive long periods (180 days) in batch systems without the requirement of host organisms. Its survival is further aided by presence of biofilms. *Legionella pneumophilia* colonization and growth in biofilms in the presence and absence of *Hartmannella vermiformis* amoeba has been studied. Green fluorescent protein-labeled (gfp) *L.pneumophila* was used for the study. Biofilms
were grown on stainless steel coupons at a retention time of 7 hours. The biofilm consisted of *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and a *Flavobacterium* species. *Legionella* levels in biofilm were observed for 15 days with and without *H. vermiformis*. It was observed that *Legionella* was unable to replicate in the absence of *H. vermiformis* but was able to persist or survive in the biofilms (33). The results indicated that biofilm was incapable of supplying enough nutrients for the growth of *Legionella* and the organisms were solely surviving.

Drinking water biofilms were grown under running tap water on different type of plumbing materials and were then spiked after 14 days with *Legionella pneumophila*. Total cell count and heterotrophic plate count of the biofilms were then monitored and *L. pneumophila* was quantified using culture based methods and culture independent fluorescence in situ hybridization (FISH) method. It was noted that after 14 days *Legionella* incorporated into the biofilms and persisted for several weeks. FISH method helped in differentiating between *Legionella* which was culturable and the ones which had entered a viable but non-culturable state (VBNC). The study was an indication that drinking water biofilms can be reservoir of *Legionella* when grown under cold water conditions on domestic plumbing materials. *Legionella* was seen to persist mostly in viable but non-culturable state in these biofilms. Bacteria enter a viable but non-culturable state when they are encountered by some form of environmental stress. When bacteria are in VBNC they fail to grow on bacteriological media via normal culture methods but they are still alive (32). Several markers such as respiratory activity, cytoplasmic membrane integrity or the presence of ribosomes indicate the VBNC state. These markers are detected by fluorescence in situ hybridization targeting 16S rRNA molecules using
oligonucleotide probes. It is still unknown whether VBNC state is induced by biofilms (36).

In 2009 the impact of biofilms usually present in water distribution pipes on the replication of *Legionella pneumophila* were studied. The biofilm similar to present in distribution system pipes were simulated on a rotating annular reactor with non-*Legionella* microorganisms like *Aeromonas hydrophila*, *Escherichia coli*, *Flavobacterium breve* and *Pseudomonas aeruginosa*. *Acantha-moeba castellanii* was added to the system. The analysis via culture and real time polymerase chain reaction (PCR) shows that biofilm associated *Legionella* bacteria increased after intracellular replication in the amoeba. After almost 48 hours replicated *Legionella* were seen to come out in bulk water from lysed amoeba cells. The study indicated the crucial role of amoeba like organisms in spread and replication of *L.pneumophila* in the distribution systems (18).

Quantitative characterization of the threshold infective dose of *Legionella* is not available due to insufficient information. The potential risks of the presence of *Legionella* in water supplies is also not fully quantified till date due to lack of suitable scientific procedures and equipment. Almost all the sources of transmission of *Legionella* to humans involve aerosolization of water contaminated with the bacteria and its subsequent inhalation. *Legionella* has been observed to be transmitted directly from the environment to humans. No vaccine is available to prevent the infection hence risk minimization is the only way by which the transmission chain between environmental sources of *Legionella* and human hosts can be broken.
The interactions between *Legionella* and other aquatic bacteria in biofilms were investigated. The possible effects of some aquatic bacteria producing bacteriocin like substances on the development and stability of *L. pneumophila* was observed. *Pseudomonas fluorescens* showed the greatest negative effect on biofilm formation and detached *Legionella* due to large amount of bacteriocin production. Other bacteria like *Pseudomonas aeruginosa, Burkholderia cepacia, Pseudomonas putida, Aeromonas hydrophila* and *Stentrophomonas maltophilia* produced bacteriocin at different levels and showed less repulsion to *Legionella* in the biofilms. *Acinetobacter Iwoffii* was seen to support and enhance *Legionella* biofilm as it did not produce bacteriocin. The results indicated that *Legionella* showed sensitivity to bacteriocin and so its production plays an important role in deciding the fate of *Legionella pneumophila* in biofilms (26).

Extracellular growth of *Legionella pneumophila* has been shown by Tison and Colleagues. *Legionella* were seen to grow on extracellular products provided by other bacteria. They discovered *Legionella* growing from an algal-bacterial mat community at 45°C in a man-made thermal effluent. Similar conditions when repeated in the lab *Legionella* was seen to grow with a cyanobacteria namely *Fisherella sp.* over a pH range of 6.9-7.6 in mineral salts medium at 45°C. They concluded that the growth of *Legionella* depended upon algal products released by the cyanobacteria after photosynthesis. Some heterotrophic bacteria were also reported to support *Legionella* growth (17).

The survival and replication of *L. pneumophila* in biofilms is still under investigation. Whether there is a necessity of a protozoan host for the *Legionella* to grow associated to biofilms or they are able to survive independently in a biofilm
community and obtain their nutrients from the environment. There is not enough
data to make a definite conclusion. There is a lack of data from pilot-scale or on-site experiments using naturally occurring consortia (1).

Understanding *Legionella*’s ecology can give important information required to build methods which prevent Legionnaires transmission through environmental dissemination. Several studies were done to understand how *Legionella* behaves in biofilms. A number of factors influence whether *Legionella* produces biofilms. Though a lot related to biofilm formation and colonization by *Legionella* has been uncovered in the last few years but there is still a lot to be explored. The presence of other microbes and physicochemical parameters has been observed to affect *Legionella* growth and colonization in biofilms and can be exploited to control its growth in water treatment and distribution system (1).

*L. pneumophila* attachment is the first crucial step when producing surface associated biofilms. Once attachment to substratum occurs a biofilm is formed. Colonization is the process of spreading and persisting within a new area. It was observed that *Legionella* was often found attached to different surfaces in the environment but its subsequent colonization dependent on a wide variety of parameters. For example the composition of the surface material to which bacteria adheres is one such important factor contributing to *Legionella* colonization. Especially materials in the plumbing like plastics were seen to support *Legionella* adherence while some others like copper were seen to inhibit its colonization. Any single disinfection technique is usually not found to be effective in totally eradicating *Legionella* due to their high resistance hence, a combination of focal and systemic disinfection techniques is needed to eliminate *Legionella* present in the
treatment train and prevent recolonization in the water distribution system. Some systemic and focal disinfection methods are hyper-chlorination, copper-silver ionization, ozonation, UV light sterilization.

**HETROTROPHIC PLATE COUNT**

Heterotrophs require organic carbon for their growth and they include microorganisms like yeasts, bacteria and molds. HPC tests give a wide range of quantitative and qualitative results based on various test conditions. HPC tests employ a range of temperature from around 20-40°C, the incubation times range from few hours to 7 days or a few weeks and nutrient conditions from low to high. Heterotrophic microorganism population in drinking water is often measured by internationally accepted test of standard plate count or culture based technique also known as heterotrophic plate count (HPC). The test originated from Robert Koch’s work in 1983. HPC does not differentiate between pathogenic and non-pathogenic microorganisms hence it gives false conclusions of health risks if based upon just the colony counts alone. HPC is an important indicator of water quality and of the performance of filtration systems. Water treated with an in line device such as carbon filter, water dispensing devices, in bottled water and in all water containing specific nutrients without residual disinfectant present experiences routine increase of HPC in water (8).

HPC recovers microorganisms which are usually a part of natural microbiota of water but sometimes they include organisms from different pollutant sources in water. A high HPC value in water samples indicates growth of microorganisms this happens especially in parts of distribution system where water is stagnant, in
domestic plumbing, carbon filters and in bottled water. Growth of microorganisms after drinking water treatment is referred to as “regrowth” which is determined based on temperature, availability of nutrients and a lack of residual disinfectant. HPC tests were therefore applied as indicators of proper function of treatment processes like filtration particularly by the end of 19th century. It is an indirect indicator of water safety. Soon in the 20th century specific fecal indicators of bacteria were adopted over HPC technique for the same.

HPC levels in drinking water varies according to the source water, the types and efficacy of treatment, the type and concentration of disinfection residuals, the age and the condition of the storage and distribution system, the concentration of dissolved organics in the treated drinking water, the ambient temperature of the raw and finished water, and the HPC method and time and temperature of incubation. HPC measurements have been crucial in making water regulations or guidelines in many countries (4). It is also used to indicate –

1) Effectiveness of water treatment processes including disinfection and so indicates indirectly pathogen removal

2) Number of organisms present as regrowth in the system which may or may not have a sanitary significance

3) Conditions of distribution system in piped systems, changes in finished water quality during distribution and storage
4) HPC levels which may interfere with coliform detection in water samples collected for regulatory compliance monitoring

5) Changes in bacterial population following treatment modifications for example change in the type of disinfectant used

6) Microbial growth potential on materials used in the construction of drinking water treatment and distribution systems.

HPC microorganisms association with health effects have been derived from epidemiological studies like outbreak investigations or risk assessments. Under the National Primary Drinking Water Regulations there is no Maximum Contaminant Level (MCL) for the HPC as a health risk, however, a limit of 500 CFU/ml in distribution system water samples is followed. This is due to the interference of high HPC levels with coliform detection for which a MCL is followed. Presence of coliform in treated drinking water in the system shows improper treatment. A level greater than 500 CFU/ml is considered to cause problems with the detection of the coliform bacteria (42). HPC microorganisms include strains of *Pseudomonas aeruginosa*, *Acinetobacter spp.*, *Aeromonas spp.*, *Klebsiella pneumonia*, and many more which are also known as “opportunistic pathogens”. Opportunistic infections were observed to be caused by some genera in the HPC these are *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Klebsiella*, *Legionella*, *Moraxella*, *Mycobacterium*, *Serratia*, *Pseudomonas* and *Xanthomonas* (38).
Studies investigating the interactions between HPC and coliform organisms were conducted in 1985. Results indicated that HPC could reduce coliform densities by almost more than 3 logs within 8 days. Some HPC were seen to injure coliform population too. Initial correlation between HPC bacteria growth and rate of coliform decline was evident. This might be due to competition for limiting nutrients like organic carbon (30).

Some of HPC microbiota were seen to regrow in water but are not detected in HPC measurements like Legionella and non-tuberculosis mycobacteria. Legionella has been observed as pathogenic to human health, though no direct relation of HPC levels to health effects or presence of pathogenic organisms have been observed (8).

Studies to quantify the concentration of heterotrophic bacteria using HPC of the water sources used by major utility and within water reaching the consumer’s taps in the City of Tucson, AZ were carried out in 2004. One of the sources was groundwater source. They collected samples of water on a monthly basis from wells at each site. The tap water of the distribution system was also sampled. A major shift from gram negative to gram-positive bacteria was observed from wells to the distribution system, to the tap. The number of HPC bacteria was seen to increase dramatically from the distribution system to the consumers tap indicating that bacteria ingested by customers is originating within the household distribution system rather than from source water or the distribution system (39).

Bargellini et al. showed a qualitative and quantitative relationship between Legionella spp. and HPC in hot water samples from different buildings. Legionella spp. counts were positively associated with HPC at 37°C using multiple regression
analysis. HPC at 22°C and 37°C was seen to have a *Legionella* concentration exceeding $10^4$ CFU/l. This suggests that HPC is an important parameter to be included in water safety plan. This association was obvious as the important requirement of *Legionella* colonization of water systems is the presence of other heterotrophic organisms which form biofilm (7).
CHAPTER 3
GENERAL MATERIALS AND METHODS

As mentioned previously the scope of this study involves the analysis of the potential growth and colonization on GAC media by HPC and Legionella in lab scale GAC media filters. The primary focus of each specific objective is to assess colonization of GAC media by Legionella and HPC and their presence in the column filtrate as measured by different analytical techniques like microscopy, HPC and other culture based techniques. These specific objectives were performed by setting up lab scale GAC column filters using tap water, settled pre-GAC filtered water, and distribution hot spot Tempe tap water. The tap water and distribution hot spot water was spiked with Legionella species to get more quantitative and qualitative analysis of the attachment, growth and colonization potential. All different water sources had various amount and types of organics which can be used by bacteria as nutrients for biofilm formation upon adsorption by GAC media particles.

WATER SAMPLE COLLECTION AND STORAGE

Water was taken from 2 different sources – Tempe tap water, settled unfiltered water from the Chaparral water treatment plant, Scottsdale, AZ

Water samples were collected from –

1) Regular Tap (ASU Campus, environment engineering lab)

2) Chaparral WTP (Scottsdale, AZ) after sedimentation (settled water) and before granular activated carbon (GAC) filtration
3) A model drinking water distribution system (MDS) pipe loop (ASU Campus, environment microbiology lab)

The model drinking water distribution system had PVC plumbing and used Tempe tap water from last 14 years (PVC-MDS). Tap water and settled unfiltered water samples were collected in 10 gallon plastic containers that were washed and triple rinsed with alconox. A volume of 10 gallon ensured extra sample of water was available from the same day and date if needed later. The containers were not treated with disinfectant like chlorine as it might have limited potential bacterial growth and colonization in the columns. Samples were stored in their collection containers at approximately 72°F prior to their use and throughout the experiment.

GRANULAR ACTIVATED CARBON PREPARATION

GAC Filtrasorb 400 is a high activity activated carbon which is manufactured from a select grade of bituminous coal, which is pulverized, re-agglomerated with suitable binder, screened to a 12 X 40 U.S. sieve particle size distribution. It was obtained from the Chaparral water treatment plant, Scottsdale, AZ which uses it for removal of dissolved organic compounds and taste and odor. The particle size was approximately 0.7mm. The GAC was rinsed multiple times, autoclaved and then soaked in sterile distilled (DI) water overnight before packing columns. Autoclaving eliminates any prior bacterial presence on the particles.
LAB SCALE MODEL WATER DISTRIBUTION SYSTEM

One out of four columns was setup with influent from the laboratory scale model water distribution systems (MDS) consisting of pipe loop shown in Figure 1: with polyvinyl chloride (PVC-MDS). The PVC loop had a main pipe that was 5.5 m long and 5.1 cm in diameter and a 1.2 m dead-end pipe, with a total volume of 50 L. The main and the dead-end pipes consisted of six and two removable sections (0.6 m in length), respectively. The main pipe was connected to a reservoir. A self-priming, thermally protected, magnetic-drive electric pump (Little Giant Pump Company, Oklahoma City, OK) continuously recirculated water between main loop and the reservoir. Pressure, flow rate (0.304 m/s), and temperature (25°C) were kept constant through external controls. Initially, the zero chlorine residual was maintained to facilitate microbial growth and biofilm formation in the pipes. To achieve 0 mg/l of free chlorine, the city of Tempe (Arizona) tap water was collected in an open tank to allow chlorine evaporation before the water was allowed to trickle under gravity to the reservoir. A turnover period of 24–72 hours was maintained for PVC-MDS. The pipe loop had been used continuously for 14 years and had well-established biofilm communities.
Figure 1. Model Water Distribution System with PVC loop and GAC media filter column

COLUMN SETUP

Larger sized glass columns straight through ends from ACE glassware were used as packing columns in three setups. The columns can withstand elevated pressures. The capacity of the column was 0.57 L and it had a diameter of 11mm. The effective length was 600mm (24 inches). The columns were setup using fittings
ordered from ACE glassware which included FETFE O-ring, screen support, retainer ring, tubing connectors, filter disc, ferrules, adaptors with flow regulator valve, and PTFE tubing–20 mm long with 4 mm thick wall. The columns were packed with Filtrasorb 400 which was washed, autoclaved and soaked in DI water overnight. Fifty grams of GAC was packed under saturated conditions occupying 240 mm of length in each of these columns. The reservoir for each column was a 20 L capacity plastic carboy. The column diameter to particle diameter ratio (or bed aspect ratio) for these columns was 16:1. Another column made of polypropylene material was used to setup GAC filter with influent from PVC-MDS. The column was 265 mm (10.5 inches) in length and 60 mm (2.5 inches) in diameter. The capacity of this column was 0.82 L. The influent pipe to this column was connected to a tap in the model water distribution system’s PVC pipe loop. Fifty grams of GAC was packed under saturated conditions occupying 110 mm length of this column. The PVS-MDS had 50 L total capacity. The bed aspect ratio for this column was 86:1.

The GAC was used as it is used in the water treatment plant and was not pulverized. This helped imitating the colonization process and growth of microbes as it would have been in a treatment plant’s large scale GAC filter. The columns were packed with a layer of glass wool first, and then glass beads and again a layer of glass wool. On top of these layers GAC bed was formed and then the columns were completely packed. The effluent of each column was recycled into the influent reservoir as the entire focus was on GAC filtration than any other unit process in the treatment train. The GAC media bed had 5 inches of room above it to collect GAC samples. The flow rate of all the columns was maintained at 10 mL/min using FMI pumps.
Figure 2. Lab Scale GAC Media Filter Columns
To sum up all the columns set up were as follows along with the abbreviation used for them in the future chapters –

a.) TWC 1 ran on tap water from the regular lab tap.

b.) WTPC ran on settled unfiltered water from the treatment plant.

c.) MDSC ran on regular drinking water moving through a model distribution system.

d.) TWC 2 also ran on tap water from the regular lab tap. It was a duplicate of TWC 1 but was started after two months of TWC 1 start date to analyze initial colonization of GAC media. This helped to run TWC 1 undisturbed for the initial one month and avoided biofilm disruption if any formed.

Four different samples were taken from each column for analysis at a time –

- Influent
- Effluent
- Column’s inner surface biofilm
- GAC particles

Only for TWC 2 biofilm samples were not taken as it was too soon after the start of the column for substantial biofilm to grow on the inner surface of the column. Out of the four samples as listed above from all the column systems influent and effluent were water samples while biofilm and GAC were solid samples. Hence, biofilm and GAC samples were converted to water samples by suspending in glycine buffer. These were then vortexed to obtain supernatant samples used for analysis further. These GAC particle samples were weighed to keep consistency in all systems which made results from different systems easier to compare.
MEDIA AND CULTURE PREPARATION

**Legionella stock culture:** Legionella stock culture was made by growing pure culture of “gfp” (green fluorescent protein) strain Legionella on BCYE plate. Inoculum from the plate was added to a 10 mL tube with DI water after substantial growth. The “gfp” strain of Legionella fluoresces under the UV due to green fluorescent protein. It was ordered from the Centre of Disease Control Prevention. The amount of Legionella in the 10 mL tube was then quantified using a spectrophotometer at 600 nm which gave an optical density reading which was then converted to Colony Forming Units per milliliter (CFU/mL). This number was confirmed using UV microscopy and by culturing the stock solution. The stock now was diluted and used to spike different column systems based on their volume.

**Phosphate Buffer Saline (PBS):** 0.5 M PBS was made by suspending 4 grams of Sodium Chloride (NaCl), 0.1 grams Potassium Chloride (KCl), 0.72 grams of Sodium Phosphate Dibasic (Na₂HPO₄), 0.12 grams of Potassium Phosphate Monobasic (KH₂PO₄) in 800mL of distilled water. The pH of the solution was then adjusted to 7.4 and the volume was adjusted to 1 L with distilled water. This solution was autoclaved at 121°C at 15 min and then used during experiments after cooling off to make HPC dilutions.

**Glycine buffer:** 0.05 M Glycine buffer was made by suspending 3.75 grams of glycine in 1 L of distilled water. The pH of the solution was then adjusted to 8.51 using 0.2 M NaOH. The solution was then autoclaved at 121°C at 15 minutes and then used during experiments after cooling off to suspend GAC and biofilm samples from the columns.
**R2A agar media:** The R2A agar media was prepared for HPC analysis during the experiments. 18.2 grams of R2A agar was suspended in 1 L of purified water and mixed well. The media was then heat treated with frequent agitation and brought to boil for 1 min until it completely dissolves the powder media. The pH of the media was checked and brought to 7.2 ± 0.2 by using 1N KOH or HCl. The media was autoclaved at 121°C for 15 minutes. The media was then cooled down to 60-80°C and poured in sterile petriplates at a volume of at least 20mL.

**BCYE agar media:** The BCYE agar media was prepared for *Legionella* analysis during the experiments. 19 g of BCYE agar base premade media mix was suspended in 1000 mL of purified water. The mixture was heated to boiling with stirring continuously to dissolve the powder. The pH of the media was then checked and brought to 6.9 using 1N KOH or HCl. The media was then put to autoclave at 121°C for 15 minutes. The media was taken out of the autoclave and cooled down to 45-50°C and maintained at it using a hotplate. L-cysteine 0.2 grams and Glycine 1.5 grams were then added to the media on a continuous stir. Inhibitor or Antibiotic solutions were mixed and added. The media was dispensed with agitation approximately 20 mL volume in each petridish (14).

**Antibiotics preparation for BCYE media:** The three main antibiotics used were Polymyxin B, Cyclohexamide, Vancomyxin. All these were stored at room temperature or at 4-20°C. Stock solutions were made in distilled water. Vancomycin stock was made at strength of 1mg/mL in 50 mL. Cyclohexamide stock was made at strength of 5 mg/mL in 50 mL. Polymyxin B was measured at 50 mg/mL strength in 10ml which was further diluted 0.5 mL in 24.5mL of DI water to make the stock.

The final amount to be added in the 500 mL autoclaved BCYE media along with L-
cysteine and glycine was 2.5 mL of the vancomycin stock, 8mL of the cyclohexamide stock and 8mL of the polymyxin B stock (14).

MICRO BIAL EXAMINATION AND ANALYSES

**HACH Spectrophotometer:** The spectrophotometer was used to detect and confirm concentration of gfp-*Legionella* strain in stock cultures which was used to spike the different column systems. Spectrophotometer scans a wide range of wavelength from 190-1100 nm and reads the sample. For “gfp” strain of *Legionella* the solution was scanned at 600 nm. A quartz cuvette is used, filled with the sample and placed in the carousel. Sample was read using an automated method of analysis for concentration in the spectrophotometer.

**High Performance Ultra Violet Transilluminator:** The Gel Logic 112 Imaging System (13 cm x 17 cm) is an electrophoresis documentation and analysis system. It was used to observe the presence of “gfp” strains of *Legionella* growing on BCYE media plates for quick detection after which they were analyzed further with other techniques for confirmation and quantification. The gfp-*Legionella* strain fluoresce green under the UV transilluminator.

**Brightfield/Epifluorescence Microscopy:** Olympus BX60 microscope, trinocular brightfield/epifluorescence was used to observe water and GAC samples for quantifying gfp-*Legionella* strain present in the samples. The strain fluoresces green under the UV light and is easy to observe. The microscope could be flipped in daylight blue color balancing filter for transmitted light path as well as neutral density filter set at 6% and 25% for transmitted light path. The stage X-Y drive was approximately 75 mm x 50 mm. Haemocytometer slide was used for observing all
types of water samples while at times microslides were also used to observe micro-
solid samples of biofilm and GAC particles directly. The observation head could be
switched between the eye and camera. Pictures were taken for certain samples with
the presence of gfp- *Legionella* strain. The slides were mostly observed under 20 X
and 40 X objectives and the eyepiece was of 10X magnification. The haemocytometer
slide was washed with 90% isopropanol and distilled water before loading samples in
the well. The samples are loaded through the wells into the slide chambers via
capillary action.

**Spread plate technique:** Based on the type of bacteria to be enumerated agar
medium was prepared and petriplates were made. The petriplates poured were
roughly 0.3 cm of thickness with a volume of 20 mL media per 100mm x 15 mm
petridish. Plates were dried in a laminar flow hood. An inoculum volume of 100-200
microliters was used on the plates. This was spread over the plate evenly using a
reusable metal spreader as some bacteria rapidly attach to the agar surface. This
metal spreader was flame sterilized by dipping in 90% isopropanol and then allowed
to cool. The spreader was placed in contact with the inoculum on the plate and
spread evenly along the length of the plate. The plate was spun continuously with
even pressure by hand until the inoculum was completely adsorbed. The plates were
set aside for few minutes and then were kept inverted in the incubator at different
temperatures according to the selective microorganism’s suitable temperature. After
incubation the plates were inspected. Colony forming units (CFUs) were enumerated
from plates having 20 to 300 CFUs. These CFUs were used to calculate the number
of CFU/mL of the original sample. When doing dilutions for HPC the plates reflected
the predictable drop in CFU/plate. Duplicates were plated and counted if the colonies were countable i.e. 20 to 300 CFU/plate.

**Scanning electron microscopy (SEM):** Carbon samples were collected from the GAC media columns and observed under SEM. Very fine particles were immobilized to a poly-Lysine media to keep them intact under the microscope. Samples were air-dried for 24 hours and adhered to 12 mm aluminum stubs using double-stick tape. Mounted samples were sputter-coated with approximately 10-12 nm of gold-palladium using a Technics Hummer-II sputter coater. Images were generated with a JSM-6300 scanning electron microscope (JEOL Inc. Peabody, MA) operated at 15kV. Scans were digitized and captured at 1024 x 1024 pixel resolution by a Model 500 digital processing unit (IXRF Systems, Austin, TX).
CHAPTER 5

QUANTIFICATION OF THE COLONIZATION OF GAC FILTER MEDIA BY

LEGIONELLA AND HETEROTROPHIC BACTERIAL CELLS

INTRODUCTION

Biofilms provide a habitat for survival and replication of Legionella species as observed in previous studies (1, 15, 17, 18, and 26). L. pneumophila have been found associated with heterotrophs in biofilms (17). Members of aquatic flora such as amoeba and protozoa play a crucial role in the life cycle of Legionella in environments. Protozoan species often feed on bacteria present in biofilms this characteristic of protozoans is exploited by Legionella to replicate. Therefore, presence of heterotrophs represents a risk factor for outbreaks of L. pneumophila in drinking water. Some studies also correlated the amount of Legionella in biofilms directly with the biomass of protozoa (52). Legionella also grows on dead cells present in such biofilms (1). The intracellular stage of Legionella within host organisms also protects it from environmental stressors (49). It was observed that biofilms produced with Legionella in presence of thermotolerant amoebae led to development of heat tolerance in Legionella species (35). Environmental biofilms contain some bacterial species which promote Legionella persistence while others which inhibit its colonization (32, 33). Flavobacterium breve and Cyanobacterial species were studied to promote Legionella growth and colonization in biofilms as they provide nutrient support (17, 26). Several experiments have also suggested the capability of Legionella to grow without the presence of protozoan species and it is
not always that *Legionella* is necrotrophic rather they are selectively necrotrophic (1).

*Legionella* is a potential threat to human health if it occurs in the drinking water and can lead to severe disease condition Legionnaires disease if outbreak of *L. pneumophila* species occurs through the drinking water treatment system or inhalation of aerosolized contaminated water (23, 27). The risk of *Legionella* passing into the distribution system increases due to its developed resistance to disinfection, biocides and other environmental stressors. However it is difficult to know that the resistance of *Legionella* in environmental biofilms is due to the biofilm structure or its association with amoeba or both. *L. pneumophila* enters a viable but non-culturable state within biofilms when exposed to environmental stresses. This makes it hard to get an accurate assessment of the contamination levels of *L. pneumophila* in systems and often requires co-culturing of *Legionella* with amoeba to lift the VBNC state (27, 36).

For long culture based techniques have been used as a standard method to quantify bacterial growth. These techniques are not so effective for assessing colonization in GAC media contactors as presence of a lot of non-specific bacteria on GAC leads to problems of over-growth of non-target bacteria in petri-dishes. Therefore, underestimating the actual concentration or number of target bacteria present in sample. Hence, other techniques such as microscopy can be relied on for a confirmation of presence or quantification of colonization and growth in GAC media filter samples.

Small scale column lab testing has been used to model the GAC media filter scenario of large contactors in facilities since the early 1980s (47). Rapid small scale
testing further was developed as a modification to predict the breakthrough behavior of larger columns based on the results of small scale columns. Columns setup as described in Chapter 3 for developing reproducible bacterial biofilms in potable water were small scale lab models which provided near realistic representation of the conditions and organisms focused in the study. The experiment was designed using these column systems. Samples from these systems were used to quantify the potential colonization of the GAC filter media by HPC and *Legionella* and their subsequent presence in the effluent using different analytical and measuring techniques.

**MATERIALS AND METHODS**

**Spiking the columns and sample analyses.** One month after the start date, Tap Water Column 1 and Model Distribution System Column (TWC 1 and MDSC) were spiked with *Legionella* using the *Legionella* stock prepared as described in Chapter 3. The one month period was given for biofilms to develop and thus convert GAC media filter into Biologically Activated Carbon (BAC) filter. The TWC 1 was spiked at a concentration of $10^4$ CFU/mL of *Legionella* in the tap water reservoir (20 L), while reservoir (50L) of MDSC was spiked at a concentration of $10^5$ CFU/mL of *Legionella*. Samples were analyzed for *Legionella* using culture based methods from the next day of spiking up till three weeks. Microscopic analyses were started after three weeks from spiking. HPC analysis was also carried out after three weeks from the day of spiking on the same days as the microscopic analysis for *Legionella*. TWC 2 reservoir (20 L) was also spiked with $10^4$ CFU/mL of *Legionella* to duplicate conditions in TWC 1. TWC 2 was spiked after it had run for a week from its start
date. TWC 2 was setup to quantify initial attachment and colonization of GAC by *Legionella* without the presence of previous biofilms on GAC particles and the column. Samples were microscopically analyzed for *Legionella* and culture plated for HPC from the next day TWC 2 was spiked up till 20 days. Samples for microscopy & HPC were collected at 5 day intervals from all the four column systems. Influent and effluent samples were collected in 15 mL tubes. GAC media samples were collected from the top of each column using a sterile 10 mL pipette. The particles were suspended in glycine buffer in 5 mL tube. Biofilm samples from column’s inner surface were taken using a swab stuck to a metal rod. Approximately 2 cm² surface area of biofilm was scrapped off the systems during each sampling event for every system.

**Legionella spread plate culture analysis.** All samples influent, effluent, GAC and column’s inner surface biofilm from TWC 1, MDSC & WTPC were plated on BCYE agar plates using the spread plate technique. The plates were inoculated with weekly samples for up till 3 weeks after *Legionella* spike to confirm the presence and absence of *Legionella* in them. Each sample was plated in duplicate. Due to excessive non-*Legionella* growth during initial analyses, latter samples were heat pre-treated for 15 min at 50°C to eliminate non-*Legionella* growth. A volume of 200 µL of each sample was inoculated on a plate at a time. This was spread over the plate evenly using a reusable metal spreader which was flame sterilized by dipping in 90% isopropanol and then allowed to cool. The plate was spun continuously with even pressure by hand until the inoculum was completely adsorbed. The plates were set aside for few minutes and then were kept inverted in the incubator at 37°C.
temperature for 3 days. After incubation the plates were inspected for the presence or absence of *Legionella*.

**Brightfield/Epifluorescence Microscopy Analysis.** For UV microscopic quantification all samples: influent, effluent, GAC and column’s inner surface biofilm were loaded directly on the haemocytometer for microscopic enumeration. The GAC samples were dipped in sterile DI water to remove any non-attached bacteria in the surrounding water on these particles. The GAC samples were weighed to get 500 mg of the sample. The swab for biofilm sample scrapped approximately 2cm² surface area biofilm during each sampling event from every system. The weighed GAC particle samples and biofilm samples were then suspended in 3 mL of glycine buffer and vortexed which helped in maximum detachment of *Legionella* from the carbon particles and biofilms.

The samples were loaded on both the ends of haemocytometer using a 10 µL pipette and observed under 20 X and 40 X magnifications. The samples were applied to the edge of the coverslip near the well and were sucked in by capillary action. The prepared slide was then observed under the microscope and *Legionella* cells present on the grid of the chamber were directly counted under UV light. The number of cells in the both the chambers’ grids were counted and averaged for a single sample. The number of cells was then converted to the concentration of cells (cells/mL or cells/cm²) in the sample as the total volume of the grid was known (1mm² = 100 nL).

The gfp-*Legionella* strain fluoresces green under the UV light and was easily detected. The haemocytometer slide was washed with 90% isopropanol, distilled water and then dried completely before loading another sample every time. Pictures were taken using a camera attached to the microscope which displayed on a
connected desktop. Results were reported as Cells/mL for all the types of samples. For GAC and biofilm samples results were also calculated and reported as Cells/mg (as shown in Appendix A) and Cells/cm² respectively.

**Heterotrophic Plate Count.** To get countable HPC the samples were diluted serially with 10% dilution and then plated on R2A media plates by spread plate technique. The dilution for all the four samples i.e. influent, effluent, glycine buffer suspended GAC and glycine suspended column’s inner surface biofilm were prepared in PBS. PBS is used for dilutions as DI water harms HPC. Different dilutions were plated for each sample. Duplicates were plated for each dilution. A volume of 100 µL of diluted sample was plated by spreading evenly over the plate. The plates were then incubated at 28°C for 5-7 days as per U.S. EPA HPC methodology (42).

**Scanning electron microscopy (SEM):** Two types of carbon samples from the operating GAC columns (TWC 1, WTPC, MDSC) were taken – washed carbon sample and unwashed carbon sample. The unwashed GAC samples were directly pipetted out from the column and observed under SEM while the washed GAC samples were first rinsed with sterile DI water. Samples were air-dried for 24 hours and adhered to 12 mm aluminum stubs using double-stick tape. Mounted samples were sputter-coated with approximately 10-12 nm of gold-palladium using a Technics Hummer-II sputter coater. Images were then generated with the SEM operated at 15kV. The samples were observed at different magnifications from 100 X to 10,000 X.
RESULTS AND DISCUSSION

TWC 1 and MDSC were spiked with *Legionella*. The influent sample from TWC 1 and MDSC was analyzed for *Legionella* concentration by culturing on BCYE media plate 24 hours later. The growth on the plate with TWC 1 sample showed order of magnitude higher colonies than on the plate with MDSC sample (as shown in Appendix B Fig. B1, B2) though the concentration of *Legionella* spiked was $10^5$ CFU/mL in MDSC and $10^4$ CFU/mL in TWC 1 reservoir. This may be because MDSC was 50 L in total capacity, which was 30 L more than TWC 1’s reservoir capacity which led to dilution of the spiked *Legionella* concentration accordingly. When GAC particles were taken out from the two columns, dipped in sterile water and directly plated on the BCYE media plates high non-*Legionella* growth was observed on the particles (as shown in Appendix B Fig. B4, B5). The high non-*Legionella* growth possibly curbed *Legionella*’s growth on the plates. High non-*Legionella* growth suggests presence of heterotrophic bacteria on the GAC particles which was later confirmed through HPC analysis.

pH of the influent and effluent from TWC 1 and MDSC was recorded a month later from *Legionella* spike. This was to confirm pH change during filtration process is within the range for *Legionella* survival.
Table 1.

pH of Influent and Effluent From TWC 1 and MDSC

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent TWC 1</td>
<td>8.3</td>
</tr>
<tr>
<td>Effluent TWC 1</td>
<td>7.7</td>
</tr>
<tr>
<td>Influent MDSC</td>
<td>8.7</td>
</tr>
<tr>
<td>Effluent MDSC</td>
<td>7.3</td>
</tr>
</tbody>
</table>

The pH ranged from 7.3 to 8.7 which was within the range for *Legionella* survival. *Legionella* survives in the range 5.5 to 9.2 (38). The pH of tap water measured was 7.4 suggesting column filters were performing in good condition.

**BCYE spread plate analysis.** Influent, effluent, and GAC were sampled from all the columns except TWC 2 and analyzed for presence or absence of *Legionella* by culturing on BCYE media plates weekly up till 3 weeks after *Legionella* spike. The plates were analyzed by naked eye observation as well as under high performance UV transilluminator. Under the transilluminator the “gfp” strain of *Legionella* fluoresced green or made the media surrounding it fluoresce making it easier to detect its presence if missed by the naked eye.
Table 2.

Presence or Absence of *Legionella* in TWC 1, MDSC and WTPC Samples Using Culture Based Analysis After a Week From *Legionella* Spike

<table>
<thead>
<tr>
<th>Samples</th>
<th>TWC 1</th>
<th>MDSC</th>
<th>WTPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Influent (<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✗</td>
</tr>
<tr>
<td>Effluent (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Effluent (<em>Leg.</em>)</td>
<td>✗</td>
<td>✗</td>
<td>✓</td>
</tr>
<tr>
<td>GAC (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>GAC (<em>Leg.</em>)</td>
<td>✗</td>
<td>✓</td>
<td>✗</td>
</tr>
</tbody>
</table>

Presence - ✓ Absence – ✗

The initial analysis showed high non-*Legionella* growth on the plates with samples from TWC 1, MDSC and WTPC. *Legionella* presence was detected in TWC 1 influent, MDSC influent, MDSC GAC sample and WTPC effluent. The presence was limited to few colonies due to high non-*Legionella* growth which overshadowed the presence of *Legionella* completely or partially. This aligns with findings from previous studies showing *Legionella* growth was curbed on culture plates due to high non-*Legionella* growth. Presence of few *Legionella* colonies was also observed in effluent from water treatment plant column (WTPC). These *Legionella* were seen to
fluoresce blue instead of green as the “gfp” strain. These were naturally occurring strain of *Legionella pneumophila*.

A heat pre-treatment for 15 minutes was applied to all the samples in 2nd week before the samples were analyzed by culture plate method. The heat pre-treatment was applied to control non-*Legionella* growth on the media plates and show clear presence or absence of *Legionella* in the samples.

Table 3.

<table>
<thead>
<tr>
<th>Samples</th>
<th>TWC 1</th>
<th>MDSC</th>
<th>WTPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent (Non-Leg.)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Influent (Leg.)</td>
<td>✓</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>Effluent (Non-Leg.)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Effluent (Leg.)</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>GAC (Non-Leg.)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>GAC (Leg.)</td>
<td>✗</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

The heat pretreatment did not give clear *Legionella* presence results and was ineffective. This was again due to very high growth of non-*Legionella* bacteria on the
plates. Just to be sure that the *Legionella* was present or if present did not enter the viable but non-culturable state (VBNC), inoculums from these plates were prepared appropriately and observed under the fluorescent microscope by loading it on a haemocytometer. The colonies from the BCYE media plates were first suspended in sterile DI water. These were vortexed and then loaded on the slide. The *Legionella* present fluoresced green under the UV microscope. *Legionella* in VBNC state is alive but does not grow on culture plates remaining undetected.

Table 4.
Presence and Absence of *Legionella* in TWC 1, MDSC and WTPC Heat Pretreated (15 min) Samples Using Fluorescent Microscopy After Two Weeks From *Legionella* Spike

<table>
<thead>
<tr>
<th>Samples</th>
<th>TWC 1</th>
<th>MDSC</th>
<th>WTPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Influent (<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>Effluent (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Effluent (<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
</tr>
<tr>
<td>GAC (Non-<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>GAC (<em>Leg.</em>)</td>
<td>✓</td>
<td>✓</td>
<td>×</td>
</tr>
</tbody>
</table>
The presence of *Legionella* was confirmed in all the samples from TWC 1 and MDSC while samples from WTPC did not show any *Legionella*. This was in accordance with the experimental design as the WTPC was not spiked with any *Legionella*.

**Epifluorescence Microscopy analysis.** Influent, effluent, column’s inner surface biofilm and GAC particles were sampled from all the columns except WTPC and were analyzed for quantification of *Legionella* under UV microscope three weeks after *Legionella* spike. TWC 2 was spiked after one week of operation and samples were analyzed 24 hours after spiking to get initial concentration of *Legionella* attachment or colonization. No biofilm samples were taken from inner surface of TWC 2 as enough biofilms had not developed during the early stage of operation. Sample was loaded on the two haemocytometer chambers and observed at 20 X and 40 X magnifications. The number of *Legionella* present on the grids of both chambers were counted and averaged and multiplied with $10^4$ to get the number of cells per milliliter for *Legionella* in the sample. Microscopic analysis was done after every 5 days. New samples were taken out on the day of analysis. The results were reported as cells per milliliter and cells per square centimeter of biofilm present over days.
Figure 3. Number of *Legionella* Cells/mL in TWC 2 Samples

*Note: Column spiked 1 week after operation, samples analysis began after 8 days*

Figure 3 shows the number of *Legionella* cells present in the influent, effluent and on GAC samples from TWC 2 over a period of initial 20 days after *Legionella* spike in the column. TWC 2 was setup to see the initial colonization or attachment of *Legionella* on GAC media. It was observed that the number of *Legionella* cells present in the influent decreased by log 0.25 (p value 0.013). This suggested that Legionella was rapidly attaching or colonizing GAC particles in the absence of developed biofilms during the initial days. The number of cells on the GAC particles started showing up 24 hours after *Legionella* spike in the column. The number of cells decreased on GAC media by 0.1 log during the initial 20 days. This
might be due to low nutrient conditions present in the tap water, shear forces due to high flow rate of water or because of a low bed aspect ratio. Number of cells in the effluent also decreased by 0.1 log over the 20 days. Number of *Legionella* cells in the effluent was higher compared to that attached on to the GAC particles which possibly is due to the low bed aspect ratio (16:1). A low bed aspect ratio of 50 or less leads to significant wall effect which has direct significant effects on the hydraulic radius – the characteristic length dimension in Reynolds number. Wall effect causes the solute molecules near the column wall to be carried along faster than the average of solutes. The wall effect also increases with the smoothness of wall surface such as glass which was the case for TWC 1 and voidage variations present along side of the walls compared to rest of the column. Hence, this might be a reason for the high number of *Legionella* cells detected in the effluent and less number of cells available to attach on the GAC particles over time. Along with the wall effect high flow rate of water through the column gave a low empty bed contact time. A low contact time of less than a minute between the water passing through and the media might be another possible reason for observing a decrease in *Legionella* cells on GAC particles in initial 20 days. A high flow rate could also lead to detachment of *Legionella* cells from the GAC particles even if attachment is detected over time.
Figure 4: Number of *Legionella* Cells in TWC 1 Samples

**Note:** Column spiked 1 month after operation, sample analysis began 3 weeks later

Figure 4 shows the number of *Legionella* cells present per milliliter and per square centimeter in samples from the TWC 1 column. The concentrations in the TWC 1 column after approximately 2 months of its operation and 3 weeks after *Legionella* spike showed a significant increase in *Legionella* on the GAC particles (p value 0.008). The number of *Legionella* cells was higher on the GAC particles after 3 weeks compared to what was during the initial 20 days (as seen in Figure 1 for TWC 2 column). *Legionella* concentration significantly increased in the biofilms (p value 0.011) developed on the inner surface of glass column. The logarithmic increase observed in column’s biofilms was 0.25. This increase in number of cells attached on
GAC particles was higher compared to that in column’s inner surface biofilm. This was in accordance with the finding that GAC adsorbs nutrients by strong adsorption forces and makes its surface favorable for increased bacterial attachment. Over time the influent water showed 0.05 log decrease in number of *Legionella* cells. This can be either due to more cells potentially colonizing GAC particles and column’s biofilms or getting inactivated. Since, it has been observed in previous studies that *Legionella* can survive for long periods of time in water it seems more likely that the decrease in the influent is due to cells colonizing GAC particles and column’s biofilms. An increasing concentration in the effluent over time could be due to high wall effect, possible exhaustion of the GAC adsorption capacity or detachment of cells due to high flow rate of water. It could also be argued that possible growth of *Legionella* in column may be released in water leading to increase of *Legionella* in the effluent.
Figure 5. Number of Legionella Cells in MDSC Samples

Note: Column spiked 1 month after operation, sample analysis began 3 weeks later

Figure 5 shows the trends in the concentration of Legionella cells in samples from the MDSC. The model water distribution system had a high amount of biofilm present in the PVC pipe loop as it had been in operation for approximately 14 years. The high amount of biofilms in the water distribution system acts as a favorable environment for Legionella cells to colonize and grow. Over days an increase in number of Legionella cells was observed in biofilms grown on the column’s inner surface (p value 0.005). The increase was an order of magnitude higher in MDSC biofilms than what was observed in TWC 1 biofilms. This difference could be due to higher bed aspect ratio (86:1) which decreases the wall effect in MDSC compared to
TWC 1 and hence increasing cell retention in the column. The roughness of the column’s surface also enhances attachment of cells to column wall as bacterial cells readily adhere to rough surfaces than the smooth surfaces. MDSC had a higher surface area covered by biofilms which is available for *Legionella* colonization then that available in TWC 1. A decrease of 0.26 logs was observed in the number of cells present in the influent which suggests their increased attachment to the column’s inner surface biofilm and GAC particles. The increase in *Legionella* concentration on GAC particles was higher (0.63 logs) than on the column’s inner surface biofilm (0.52 logs). The number of cells attached to column’s inner surface biofilm was much higher than that on to GAC particles as the entire pipe loop was excessively colonized by biofilms. The bed aspect ratio between MDSC and TWC 1 may have also contributed to less number of cells detected in the effluent from MDSC compared to the TWC 1 effluent. The number of cells in the effluent but increased three times from what it was initially. It was difficult to predict whether the increase in number of *Legionella* cells in the system was because of possible growth or just increasing attachment as cells were not detected in a high number in the effluent from MDSC. If cells are not growing they could have also entered the viable but non-culturable state and might just be attached.
**Heterotrophic plate count analysis.** HPC concentrations in influent, effluent, biofilm on column’s inner surface and GAC samples were also studied from all the columns and results were reported as HPC per milliliter and HPC per square centimeter. TWC 2 was spiked and samples were analyzed 24 hours after the spike to get initial concentrations of HPC attachment and colonization. Biofilm samples from inner surface of TWC 2 were not analyzed as it was too early in operation for biofilms to substantially develop in the particular column. Presence of HPC strongly indicates the potential survival and presence of *Legionella* in the system and in the samples. *Legionella* has been observed to inhabit biofilms formed by heterotrophic bacteria in a GAC filter system for nutrients and survival. *Legionella* sometimes only persists in the presence of HPC and loses the ability to grow becoming VBNC. Countable numbers of colony forming units of heterotrophic bacteria in different samples were determined by plating different dilutions of each sample on culture plate. The plates were incubated at 28°C and CFUs were counted after every 5-7 days. HPC was analyzed on the same day as the microscopic analysis using the same samples.
Figure 6. HPC/mL Present in Samples From TWC 2

Note: Column spiked 1 week after operation, samples analysis began after 8 days

Figure 6 shows the HPC in TWC 2 samples over a period of 15 days after *Legionella* spike in the column. The number of HPC in the influent showed an increase of 0.21 logs indicating growth and multiplication of heterotrophic bacteria (p value 0.019) in the reservoir. There is maximum nutrients in the reservoir water during the first 15 days as all of it had not yet been filtered through the column. The increase could also be due to the recycled effluent which adds HPC to the influent. The number of HPC on the GAC particles was observed to increase overall by 0.34 logs. The increase in the attached heterotrophic cells was interspersed by reduction in the HPC which could be reasoned due to wall effect leading to the
release of cells in the effluent instead of getting attached on to the GAC particles. Along with the wall effect high flow rate of water through the column might have caused detachment of heterotrophic bacteria from the particles. The number of cells detected in the effluent was low compared to the number present in the influent and that attached on the GAC particles indicating increased attachment and potential colonization of the particles by heterotrophic bacteria over initial 15 days.

Figure 7: HPC Present in Samples From TWC 1

Note: Column spiked 1 month after operation, sample analysis began 3 weeks later

Figure 7 shows heterotrophic bacteria to be growing in the order of $10^7$ HPC/mL in the TWC 1 column. The GAC particles showed a gradual overall increase of 0.16 logs in the HPC over the course of a month suggesting growth and
colonization of bacteria on the particles. This observation coincides with the increased *Legionella* concentrations seen on the GAC particles during the same time (Figure 2). The HPC in the influent decreased by 0.002 logs over the period of an entire month. This small decrease overall might be due to the reduction in cell number because of death being almost equal to amount of increase due to recycled cells and potential growth over a month. The effluent decreased by 0.21 logs in the HPC indicating increased attachment and colonization on GAC particles by heterotrophs over time. High concentration of HPC in the system on GAC and column’s inner surface biofilm could be the reason why high *Legionella* concentrations were also observed during the same time in the same samples (as seen in Figure 2). Any number of heterotrophic bacteria in the effluent might be due to higher wall effect caused by lower bed aspect ratio as well as smooth glass surface of the column. The detected cells also indicate possible exhaustion of GAC adsorption capacity over time or sloughing off of bacteria attached to biofilms. Microscopic analysis of effluent samples over time shows number of *Legionella* cells detected gradually increased by 0.38 logs (as seen from Figure 4) while HPC gradually decreased by 0.21 logs. This shows relationship between the heterotrophic bacteria and *Legionella* survival in the GAC media filter columns. The HPC increase or decrease in all samples was not highly significant at this point of time approximately 2 months after column operation suggesting the columns might have reached a stable state of filtration and GAC possibly started getting exhausted of its adsorption capacity.
**Figure 8**: HPC Present in Samples From MDSC

**Note**: Column spiked 1 month after operation, sample analysis began 3 weeks later

As seen in Figure 8 above the HPC was observed to increase significantly overall in the column’s inner surface biofilm over days. This was expected due to higher surface area covered by biofilms in the system. The increase in cells observed in the biofilm samples of MDSC was higher than that in TWC 1 inner surface biofilm samples. This is due to higher wall effect in TWC 1 leading to lower retention of cells in the column system. Increase in HPC observed on GAC particles (log 0.20) from the MDSC system was higher than the increase of cells in column’s inner surface biofilm (log 0.15). The GAC particles and column’s inner surface biofilm showed a significant increase in the HPC indicating attachment and potential
colonization (p value GAC particles 0.007; p value column's biofilm 0.009). The influent and effluent HPC decreased by 0.03 logs and 0.02 logs respectively. This suggests that HPC decreased in the influent and effluent over time and started attaching and colonizing on the GAC particles and column's inner surface biofilm or possibly cells were getting inactivated due to lack of nutrients and competition in both the samples. Presence of HPC detected in the effluent might also be due to sloughing off of cells from the biofilms by a high flow rate of water through the system. The log increase in *Legionella* cells detected in the MDSC effluent (as seen in Figure 3) was 0.60 while the decrease in HPC was 0.03 logs. The detection of HPC and *Legionella* in effluent in varied concentrations could be due to growth of HPC and *Legionella* both but it is hard to confirm this in the complex system.
Figure 9. HPC Present in Samples From WTPC

Note: Samples analyzed approximately 2 months after operation

Figure 9 shows HPC were present in the order of $10^8$ HPC/mL in the WTPC. This is because of partially treated water used in the filter system. HPC present in the biofilms developed on column glass and on the GAC particles from the WTPC was observed to increase though not significantly. This suggests attachment and colonization or possible growth. The HPC in the influent decreased by 0.02 logs while that of the effluent increased by 0.07 logs during the third month of WTPC operation. The influent HPC decreased suggesting increased attachment and colonization on the GAC particles and column’s inner surface biofilms. The effluent HPC increased which could was due to a high wall effect based on the bed aspect.
ratio (16:1). The column surface being smooth and made up of glass increased the wall effect further. The increased effluent concentration over time also indicates sloughing off of the bacteria from attached biofilms or increased growth in the system. The water used in the WTPC is from water treatment plant after sedimentation and before GAC filtration which probably already had a high concentration of HPC as it was not fully treated.

**Scanning Electron Microscope Analysis.** The GAC samples from all the columns were dipped and rinsed in sterile DI water. The washed GAC grains were then transferred to 5 mL tubes containing sterile DI water and were taken to microscopy facility for SEM imaging. The images showed presence of heterotrophic bacteria and biological activity in TWC 1 and MDSC GAC particle samples confirming presence of HPC on the particles and that *Legionella* can attach and colonize these surfaces. Diatom was observed in one of the samples from TWC 1 which is both heterotrophic and autotrophic but the water species are mostly heterotrophic. High colonization was not observed on GAC particles as samples were always taken from top of the columns after every 5 days disturbing any potential colonization process. Samples from increased depths which are not disturbed and hence, might show higher colonization on GAC particles were not accessible for sampling due to column design. Also, the run time for these columns was not substantial for building up heavy biofilm colonization.
Figure 10. Presence of Heterotroph on GAC Surface From TWC 1

Figure 11. Presence of Biological Activity on GAC Surface From MDSC
Figure 12. Presence of *Legionella* in a TWC 1 Sample From a BCYE Media Plate

Figure 13. Presence of *Legionella* in MDSC Sample From a BCYE Media Plate
CHAPTER 6

CONCLUSIONS AND CONSIDERATIONS

The results from different experiments in this study appear to align with findings previously published in literature. The trends during the initial days of the column operation (TWC 2) show attachment of *Legionella* on GAC particles 24 hours after *Legionella* spike. During initial 20 days biofilm formation had just begun and hence the number of *Legionella* present on the GAC particles showed reduction of log 0.1 over time. The number of *Legionella* cells in the influent and effluent detected at this time was higher than that attached on particles. This was due to low bed aspect ratio (16:1) which may have resulted in high wall effect or channelization of solutes near the wall of the column than through the GAC media. This could also be due to high flow rate and low contact time available to the cells to attach. The initial attachment could be due to just physical retention on the GAC media which leads to subsequent steps in biofilm formation. On the other hand the heterotrophic bacterial count was observed to increase on the GAC particles initially which could be due to physical retention, attachment and potential colonization. Attachment being the first step of biofilm formation indicated slow development of biofilms over time in the columns and on the GAC particles. One month after *Legionella* was spiked in columns (TWC 1 and MDSC) its presence was confirmed on all the samples collected from these columns. An increase in the attachment and potential colonization on column’s inner surface biofilm and GAC particles by *Legionella* was determined by comparing each time point with the initial phase when enough biofilms had not developed in the system (TWC 2).
The number of *Legionella* cells (by microscopy) and heterotrophic bacterial cells (by cultural-based method) detected in samples from all columns after approximately two month of their operation indicated significant increase in number of cells present in column’s inner surface biofilms and on GAC particles. The increase in *Legionella* on GAC particles were greater (0.42 logs in TWC 1 and 0.63 logs in MDSC) than the biofilm grown on column’s inner surface (0.25 logs in TWC 1 and 0.52 logs in MDSC). Though, the log increase was higher on the GAC particles but the number of cells attached was higher on the column’s inner surface biofilms. This suggests *Legionella* attaches and grows well in support of biofilms if present in the system. The influent concentration of *Legionella* and heterotrophic bacteria decreased while that of the effluent was seen to increase after two months in TWC 1 and MDSC. The increase in the effluent could be related to the different bed aspect ratio which resulted in different level of wall effect in the two columns. The wall effect was higher for TWC 1 compared to MDSC. High effluent concentrations also indicated that over time GAC was probably getting exhausted of its capacity to sustain biofilm and *Legionella*. The samples were also tested for *Legionella* by culture-based technique using BCYE media plates but it did not indicate presence of *Legionella*. This was either due to high non-*Legionella* growth overshadowing presence of *Legionella* or due to *Legionella* entering viable but non-culturable state. In viable but non-culturable state *Legionella* persists but loses the ability to grow.

It was observed that increase in heterotrophic bacteria on the GAC particles and in the column’s inner surface biofilms coincided with an increase of *Legionella* attachment and colonization during the same time. This was in accordance with the characteristics of *Legionella* to coexist and grow on established biofilms. Also,
Legionella was detected to increase in the effluent of columns during the time when HPC was observed to decrease in effluent. This suggests sloughing off of Legionella cells from biofilms due to shear forces by high flow rate of water in the columns. Another possible explanation of increased Legionella concentrations in effluent could be its growth in the column or possible exhaustion of GAC adsorption capacity over time.

To interpret the data and further investigate some of the results of this study, future considerations should be made. First, use of only one single technique to quantify Legionella cells in GAC media filter system could give inaccurate results. For example, culture based analysis method used initially to quantify cells was inaccurate due to high non-Legionella growth detected on the BCYE plates. Heat pre-treatment applied to samples for 15 min was also not effective in eliminating non-Legionella growth from plates suggesting use of only culture based analysis method would give inaccurate quantification. Cells might also enter the viable but non-culturable state where they persist but lose the ability to grow and remain undetected on these media plates. The results vary in such cases from the actual number so other techniques like epifluorescence microscopy could be used to detect and confirm such living but non-multiplying cells in the samples. It gives clear countable numbers for the Legionella cells present per milliliter in samples. It is observed that green fluorescent protein containing strain of Legionella could lose its fluorescence under UV over time and lead to inaccuracy in counting number of cells present in a sample under epifluorescent microscope. Hence, use of two methods together is better to reduce quantification errors in complex systems like GAC filters.
Second, the use of tap water as in this particular study could give considerably less colonization on GAC particles as the amount of nutrients and organics present in tap water are less to sustain a large number of bacterial cells. Tap water is treated and if recycled it gets deprived of any available nutrients. Therefore, future studies using water samples containing higher organic concentration would give a broader picture of colonization on GAC particles by bacteria along with decrease in the organic content of the water sample. The normal notion of biological growth on GAC media being beneficial is not always true as seen from the study. Colonization of opportunistic pathogen on biofilms established by other beneficial bacterial colonies could lead to their subsequent detection in the filtrate causing disease outbreaks.
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APPENDIX A

GRAPHS OF GAC SAMPLES
Figure A1. Number of Legionella Cells/mg in GAC Sample From TWC 2
Figure A2. Number of *Legionella* Cells/mg in GAC Sample From TWC 1
Figure A3: Number of *Legionella* Cells/mg in GAC Samples From MDSC
Figure A4. HPC/mg in GAC Sample From TWC 2
Figure A5. HPC/mg in GAC Sample From TWC 1
Figure A6. HPC/mg in GAC Sample From MDSC
Figure A7. HPC/mg in GAC Sample From WTPC
APPENDIX B

IMAGES OF BCYE CULTURE PLATE ANALYSIS FOR LEGIONELLA
Figure B1. Legionella Presence in MDSC Influent Sample
Figure B2. Legionella Presence in TWC 1 Influent Sample
Figure B3. Fluorescent gfp-strain of *Legionella* Glowing Green Under UV Transilluminator

(Left - Media as well as cells glow green, Right – *Legionella* cells glow green while non-*Legionella* does not under UV light)
Figure B4. Difference Between Growth on BCYE Plates With GAC Particles Versus GAC Particle Supernatant Plated From MDSC

(Left: Growth of Non-\textit{Legionella} around GAC particles from MDSC, Right – Growth of \textit{Legionella} from GAC particle supernatant after vortexing and plating)
Figure B5. Difference Between Growth on BCYE Plates With GAC Particles Versus GAC Particle Supernatant Plated From TWC 1

(Left: Growth of Non-Legionella around GAC particles of TWC 1, Right – Growth of Legionella from GAC particle supernatant after vortexing and plating)
APPENDIX C

UV MICROSCOPY IMAGES
Figure C1. Legionella Attached to GAC Fines
Figure C2. *Legionella* Under UV Microscopy Attached to GAC Particle Sample From TWC 1
Figure C3. *Legionella* Under UV Microscopy Attached to GAC Particle Sample From TWC 1
Figure C4. Legionella Under UV Microscopy Attached to GAC Particle Sample From MDSC
Figure C5. *Legionella* Under UV Microscopy Attached to Biofilm Sample From MDSC
APPENDIX D

SEM IMAGES
Figure D1. Presence of Biofilm on Surface of GAC Particles From TWC 1
Figure D2. Biofilm Like Formation on Surface of GAC Particles From MDSC