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Retention and Removal of Bacterial Endospores from Common Drinking Water Distribution System Pipe Materials

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Abstract

Biological contamination with pathogens, whether intentional or unintentional, is a potential problem in drinking water distribution systems. This study assesses the possibility of spore-forming pathogens retention on internal drinking water distribution system pipe surfaces. In the first study, polyvinyl chloride (PVC), cement-lined ductile iron, and ductile iron pipe coupons (3 cm x 14 cm) cut from new water main piping were conditioned for three months in dechlorinated Cincinnati, Ohio tap water. Bare and conditioned coupons were spiked with *Bacillus atrophaeus* subsp. *globigii*, a surrogate for *Bacillus anthracis.* Spore retention differed between pipe materials and the presence of established biofilm from conditioning also had an impact. Conditioned PVC coupons had significantly lower spore retention (31±11%) than conditioned cement-lined coupons (61±14%) and conditioned iron coupons (71±8%). In addition to measuring spore retention, two sampling techniques, brushing and scraping, were tested for their ability to recover the inoculated spores from the coupons. Mean recoveries for all materials ranged from 37±30% to 43±20% for brushing vs. 24±10% to 51±29% for scraping. On cement-lined pipe, brushing yielded a significantly higher recovery than scraping. No differences were seen between brushing and scraping the PVC and iron pipe coupons.

The second phase of experiments involved comparisons of the three pipe materials with different surface preparations – bare (new), conditioned (exposed to flowing city tap water for 13 months), and coated with artificial “biofilm” (agarose). It should be noted that in this case, the conditioning tap water was used as delivered from the tap and was

not dechlorinated. To assess spore retention on the various surfaces, a suspension of $2x10^4$ spores in 2 mL of dechlorinated tap water was applied to the coupon surfaces for 20 minutes followed by rinsing with dechlorinated tap water to collect spores to determine the number of spores released from the surface. Membrane filter plate count numbers were used to enumerate spores released from the coupon. Due to non-normal distributions, all data were analyzed using non-parametric statistics. Conditioned ductile iron retained significantly more spores than the other pipe materials as seen by the significant differences between conditioned PVC, conditioned cement, bare iron, and agarose-coated bare iron. Significant differences were also seen between conditioned cement and conditioned PVC. A second study was conducted to evaluate spore retention on artificial "biofilm" vs. a natural, but nutrient augmented "natural" biofilm grown in a recirculation tank. The second study was done at three different spore concentrations. Conditioned ductile iron retained significantly more spores than the other pipe materials as seen by the significant differences between the condition iron and: conditioned PVC, conditioned cement, bare iron, and agarose-coated bare iron. Significant differences were also seen between conditioned cement and conditioned PVC. Tests comparing spore retention between agarose "biofilm" and a nutrient-augmented biofilm also showed that for all three concentrations, augmented "natural" biofilm significantly retained more spores than agarose "biofilm". Augmented "natural" biofilm on PVC coupons retained more spores than agarose "biofilm" on PVC. In contrast, augmented compared to agarose biofilm on cement-lined coupons showed no significant differences leading us to believe that the agarose "biofilm" simulates spore retention as long as the natural biofilm covers the pipe surface at a certain thickness.

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CHAPTER 1: BACKGROUND

1.1 Introduction

In the past decade, especially following 9/11 and the anthrax mailings, the possibility of persistent biological agents being used to shut down critical infrastructure has become an increasing concern. Many persistent biological agents require extensive and lengthy cleanups once they are distributed into the environment and, therefore, they have the potential to disrupt public health and the economic vitality of an area. Drinking water systems are susceptible to intentional contamination due to their complexity and the many components that make up the system such as water storage tanks, storage reservoirs, pumps, etc. (Clark & Deininger, 2000). Furthermore, the different internal surfaces that are present in a distribution system provide a substrate for persistent contaminants to adsorb or adhere to. Biofilm covers many of the internal surfaces in a drinking water distribution system and will be in close contact with the water. If a persistent contaminant is adhered or absorbed into the biofilm, it can detach over time due to natural sloughing or changes in flow within the system. It may then be present in the water phase in concentrations that may be hazardous. Thus, persistence of the contaminant and retention of the contaminant on the internal surfaces of the system are important considerations; especially due to the requirements for decontamination after the contaminated water is flushed from the system. Lengthy and extensive cleanups will be required before the system can be returned to a pre-contamination status. In addition to sampling, it is important to understand potential levels of adhesion of organisms to the internal surfaces.

Persistent pathogens require extensive and lengthy cleanups once they are distributed into the environment, and therefore, they have the potential to disrupt public health and the economic vitality of an area.

Bacillus spores are recalcitrant and may remain viable in water for at least two years even in the presence of increased levels of chlorine (Maginnis *et al.*, 2001; Watson & Keir, 1994). Spores have also been shown to be retained on water infrastructure surfaces (Szabo *et al.*, 2007). Taking into consideration the spore's persistence and ability to adhere to surfaces, it is important to assess how to accurately sample surfaces of water infrastructure in order to determine the extent of contamination as well as the efficacy of the decontamination of the system. There are currently protocols for collecting bulk water samples from a distribution system if contamination is suspected. However, there are no documented standardized methods that cover surface sampling of the internal surfaces of drinking water infrastructure for contamination.

Characterizing bacterial retention on internal surfaces of drinking water distribution systems is a complex endeavor. It requires taking into account the physical and chemical characteristics of both the bacteria and the internal surfaces of the system. Water parameters such as pH, ionic strength, and disinfectant residual influence the physical and chemical interactions within the system; which either increase or decrease bacterial retention. Biofilm and corrosion buildup throughout the system will also play a role in retaining bacteria and other contaminants. Lastly, it should be recognized that the variations in the flow of water through the system will impact the parameters mentioned

above adding further complexity. In cases where organisms adhere to pipe walls or other components of a distribution system, decontaminating the surfaces and estimating the level of retention on the surfaces become important in returning the system to normal use. Therefore, assessing the contamination potential of common drinking water pipe materials as well as techniques for accurately measuring bacterial surface contamination are important to investigate.

1.2 Bacillus spores

Bacillus anthracis is a gram positive bacillus. Its endospores are metabolically inactive hydrophobic spheres around 3 µm in diameter. While *Bacillus anthracis* spores are not endemic to drinking water systems, they represent a substantial threat if they were intentionally introduced. Animals and people can be exposed to *B anthracis* through three primary routes; inhalation, contact with the skin, and ingestion (Dragon & Rennie, 1995). Due to its past use as a bioterrorism agent and especially after the anthrax mailings in 2002, spores are perhaps the most well known CDC Category A pathogen (Darling *et al.*, 2002).

Vegetative Bacillus cells do not last long in harsh environments. Therefore, sporulation occurs and protects the organism if the spore comes in contact with environmental stresses such as high temperatures, direct sunlight, desiccation, starvation, etc. (Dragon & Rennie, 1995). Spores are metabolically dormant; however, when receptors on the spore coat come in contact with certain nutrients such as mixtures of glucose, fructose, and asparagines mixed with water, the spore germinates immediately

and becomes a vegetative cell (Setlow, 2003). Once a viable *Bacillus anthracis* spore makes contact with a nutrient rich environment such as a lung or gastrointestinal tract, the spores germinate and replicate rapidly. The bacilli then release toxins that lead to hemorrhage, edema, and necrosis. The spore outer layers consist of an inner and outer coat layers with outer spore coat that is morphologically complex. The outer coat, also called the surface layer, consists of several layers of peptidoglycan sheeting. The inner coat also consists of a thinner layer of proteinaceous material (Fernando & Othman, 2006). Fernando & Othman also suggest that the *B. subtilis* spore coats provides resistance to disinfectants due to the low permeability of hydrophilic molecules.

While spores can survive up to 40 years in dry climates and can be harmful through the inhalation route, they are also recalcitrant in water, even in the presence of biocides. *Bacillus anthracis* spores are not endemic to drinking water systems, but they represent a substantial threat if they were intentionally introduced. While there are multiple spore forming *Bacillus* species that could be used as surrogates for *Bacillus anthracis*, *Bacillus atrophaeus (globigii*) is the same size and morphology and has been found to be more resistant to chlorine than the more virulent species of *Bacillus* (Brazis *et al.*, 1958). With respect to spore adhesion, it has been shown, that *Bacillus* spores of different species tend to exhibit differences in their ability to adhere to surfaces depending on the strain (Faille *et al.*, 2002). Therefore, the strain used in this study, *Bacillus atrophaeus (globigii*), may not completely represent *Bacillus anthracis* with respect to its affinity for clumping or adhesion in water, but it is thought to behave more conservatively and serves as a practical non-pathogen surrogate.

Predicting retention of *Bacillus* spores on pipe and biofilm surfaces is another important factor since it has been shown that attached biofilm organisms are more resistant to disinfectants than the same organism in its planktonic form (Meyer, 2003). Biofilm and surface roughness from corroding pipe materials provide substrates where spores can be retained and where they can survive despite high concentrations of disinfectant in the bulk water. The interaction of various mechanisms of attachment and retention to pipe surfaces can account for survival of spores in water with high disinfectant concentrations. Furthermore, *Bacillus* spores tend to adhere to surfaces up to 10 fold more than vegetative cells of the same species (Flint *et al.*, 2001). Therefore, impacts from a contamination event are prolonged when retained spores remain viable and detach into the water over time. Due to the multitude of factors that go into determining an infectious dose for pathogens, there are no complete studies on oral reference doses, or no effect levels for anthrax, however, under the Safe Drinking Water Act, the non-enforceable Maximum Contaminant Level Goal (MCLG) has been established as zero for pathogens in all water systems.

As an example of a microbial contaminant in drinking water, the Surface Water Treatment Rule in 1989 established treatment requirements for 99.9 percent and 99.99 percent removal for Giardia and viruses respectively. Regulators made these decisions based off of an assumption that there would be an annual risk of no more than 1 infection per 10,000 people exposed over a year from drinking water if the regulations were implemented. Similar risk based decisions would have to be made in the event of

intentional contamination of a system. Some studies have suggested that human exposure through ingestion may be harder to diagnose than dermal or inhalation anthrax. This is due to the inability to correctly diagnose the disease and provide medical intervention earlier on, which may lead to increased mortality (Inglesby *et al.*, 2002).

1.3 Physiochemical Mechanisms of Bacterial Adhesion

In the event that spores are introduced to a drinking water system, they can either remain planktonic, be passively transported within the system, or they could adhere to a surface or other particles within the bulk water. In either case, there will be multiple interactions influencing the transport of spores or adhesion of the spores to a surface or other particle. Similar numbers of spores introduced into the bulk water in the system in a short amount of time may create different levels of contamination and adhesion than spores that were injected for a longer time period.

The most important factors influencing the fate and transport of a spore, or any microbe, in a distribution system, can be divided into four categories: Flow within the system, internal surface characteristics including presence or absence of biofilm, physical and chemical characteristics of the spore (additionally, particle size, hydrophobicity, charge or electrokinetic potential, cell concentration, species, and culture conditions), and the chemical properties of the water such as the pH, viscosity, and temperature of the medium. Thus, adhesion in a liquid medium has been shown to be a complex process and depends on multiple parameters which can dictate the level of adhesion (van Loosdrecht

et al., 1990). The relative influence of these groups of characteristics on where and in what condition the spore ends up would be hard to predict due to the interactively complex nature of the system. Therefore, it is difficult to estimate which factors or factor has the most impact on a spore within the system.

1.3.1 Hydrophobicity

A positive correlation has been shown to exist between adhesion of drinking water bacteria and the surface hydrophobicity of the pipe material, making hydrophobicity an important parameter influencing bacterial adhesion to pipe. Due to the fact that spores, to varying degrees depending on the species, are highly hydrophobic, hydrophobicity is one of the more important parameters that can be measured and correlated with adhesion. Higher numbers of bacteria have been found to adhere to pipe surfaces as the surface hydrophobicity of the bacteria increases (Simoes *et al.*, 2007). Common ways to determine hydrophobicity include hydrophobic interaction chromatography (HIC), 2 phase partitioning, microbial adhesion to hydrocarbons (MATH), atomic force microscopy, and contact angle measurements.

The basic approach for quantifying hydrophobicity, suggested by Absolom (Absolom *et al.*, 1983), implies that bacterial adhesion will be favored if the process causes the free energy in the system to decrease. Absolom illustrates this free energy balance using the following formula:

$$\Delta F^{adh} = \gamma_{BH} - \gamma_{BL} - \gamma_{SL}$$

Where F^{adh} is the free energy of adhesion, γ_{BH} is the bacterium-substratum interfacial tension, γ_{BL} is the bacterium-liquid interfacial tension, and γ_{SL} is the substratum-liquid interfacial tension. Energies of adhesion include hydrophobic interactions between the bacteria and the surface in addition to Van Der Waals forces which can influence bacterial adhesion.

Bacterial endospores tend to be more hydrophobic than vegetative cells of the same bacteria. To illustrate this, Husmark *et al*. found *B. cereus* endospore adhesion to stainless steel was 10 times greater than that observed for vegetative *B. cereus* cells (Husmark & Ronner, 1990). The difference in hydrophobicity between vegetative cells and spores involves the morphological and chemical differences between the spore coat and the vegetative cell coat. According to Husmark and Ronner, around a pH of 3, spores were more likely to attach to a stainless steel surface due to a neutral charge. pH values above 3 and below 3 resulted in decreased adhesion. Additionally, it was found that spore adhesion to a hydrophobic surface increased six-fold as ionic strength was increased with $MgSO_4$. The addition of NaCl to the spore suspension resulted in a four-fold increase in adhesion.

In contrast to the changes in spore adhesion related to ionic strength, both Van Der Mei *et al.* (Van Der Mei *et al.*, 1998) and Van Loodsrecht *et al.* (van Loosdrecht, *et al.*, 1990) suggest that hydrophobicity is the dominant characteristic in bacterial adhesion. Accordingly, electrokinetic potential mostly influences cells with low hydrophobicity.

Van Loodsrecht *et al.* showed that most of the bacteria that had hydrophobic cell walls also had higher negative charges but also explained that highly hydrophobic cells with low charges would be hard to isolate from environmental samples due to bacterial adherence to particles or other bacteria within the sample.

1.3.2 Electrostatic Forces

Bacterial cell surfaces possess acidic and basic functional groups contained in the cell walls, lipopolysaccharides, phospholipids, and proteins. These materials exhibit positive and negative properties which make the bacteria either positively charged or negatively charged. For example, carboxylic acid functional groups exhibit a surface charge which is dependent on the state of ionization of the functional group i.e. COO^- vs. COOH. The charges of the particle or bacteria are therefore, dependent on the pH and ionic strength of the water surrounding the cell surface (Hong & Brown, 2006).

The free energy of adhesion demonstrated by Absolom is influenced by different physiochemical mechanisms. In examining the charge of a bacteria suspended in water and the impacts of its charge on adhesion, it is important to understand the interactions between particles and other particles and surfaces as described by the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory which was developed it in the 1940s. DLVO theory describes the force between charged surfaces interacting through a liquid medium. According to the theory, the sum of the interactions between two particles will be equal to the sum of the attractive Van Der Waals forces minus the repulsive force of the charged particle. Since bacteria are organic, the majority will have a negative charge at a

natural pH, which, when in a liquid, creates a layer of ions around the cell. Higher ionic strengths of the liquid decrease this layer, due to counterions entering the layer. This reduction in size of the layer increases the chances that electrostatic repulsion of like charges can be overcome and Van der Waals forces can take effect, resulting in the two particles adhering to each other. This theory can be taken a step further to include electrostatic interactions between a particle and a surface. According to Tidswell (2005), at around 10 nm from a surface, a bacterium will experience a predominant force of attraction. Within 5 nm, the bacteria will experience electrostatic repulsion that will predominate until about 2 nm, at which point the surface Van Der Waals forces secure the bacterium at the surface (Tidswell, 2005). These electrostatic forces are the key drivers in the traditional water treatment process of coagulation where raw water is mixed with coagulant, which reduces the electrostatic repulsion between particles and allows them to coagulate.

Zeta potential represents the charge of a suspended particle and represents the magnitude of the charge on the particle surface. This parameter is particularly important in that it relates to the degree of repulsion or attraction a suspended particle in a liquid has with other particles or with charged surfaces. Particles in suspension that have higher zeta potentials will be more stable and therefore, will not adhere to each other or surfaces. Alternatively, particles that have lower zeta potential will tend to attract each other, form flocs, or adhere to surfaces. The most common zeta potential measurement method consists of placing bacteria in an electric field and then detecting movement of the particle or bacteria in the field. Thus, with this method, zeta potential is not measured

directly, but is calculated indirectly by determining the movement of the particle and associating that with a zeta potential.

Lytle *et al.*, tested the electrokinetic properties of various bacteria to determine how the surface charge was impacted by pH and ionic strength. It was found that different bacteria assessed at different electrostatic charges differ greatly given different water chemistry conditions (Lytle *et al.*, 2002). In the study, ionic strength of various suspensions was increased by the addition of potassium phosphate buffer to the water. The pH was increased by adding NaOH or HCl to the suspensions. As the ionic strength increased, it was found that the electrophoretic mobility (EPM) also increased for all the microorganisms tested. The study showed that as the pH went up, the EPM increased relatively sharply up to a pH of around 5-6. The zero point of charge (ZPC) occurred between 2 and 4 for the bacteria that were tested. Additionally, the results showed that as the concentration of buffer went up, the EPM increased.

Due to the complexity and the many different physicochemical mechanisms and biochemical mechanisms within the cell, it is evident that there is not one mechanism that should be used to predict bacterial adhesion since it is the combined interactions of different forces and cell characteristics that cause bacteria to adhere (Bos *et al.*, 1999).

1.4 Biofilm

It has been estimated that 95% of the overall biomass in a distribution system is attached to walls (Block, 1992) The presence of biofilm, along with corrosion, on internal

distribution system surfaces makes studying and predicting bacterial retention to surfaces more challenging. Furthermore, biofilm and corrosion are known to slough off over time or when there is a fluctuation of water movement in the pipes, releasing contaminants that may be integrated into the biofilm. The biofilm consists of a layer of viable cells coated with polysaccharides and excreted cellular products which protect the community of bacteria and enhance its ability to remain viable and attached to the surface despite the presence of disinfectants and shear from water movement along the pipe wall (Sutherland, 2001b). The presence of biofilm may also increase or decrease the affinity for pathogenic bacteria or spores to adhere (Flemming *et al.*, 2002). Distribution systems contain different amounts of nutrients as well as different hydraulic characteristics which will influence the level of biofilm growth. Hydraulic conditions and pipe materials may make the distribution of biofilm irregular, making it difficult to define a "standard" drinking water biofilm. Despite the different biofilm levels in drinking water systems, Ollos *et al.* stated that a bacterial cell count of 10^4 CFU/cm^2 or higher is considered to adequately represent a viable drinking water biofilm (Ollos *et al.*, 2003).

The complex process of bacterial adhesion and, in the case of biofilm level of colonization, also depends on the pipe material which provides the substrate for adhesion and colonization. The three most commonly used pipe materials in the United States (PVC, iron, and cement) exhibit different bacterial regrowth characteristics. For example, in studying differences in biofilm formation on pipe coupons, it was found that iron pipe had the highest regrowth potential, followed by cement, and PVC was the lowest (Camper *et al.*, 2003).

Prior to planktonic cells adhering to a solid substrate, a conditioning film, which is comprised mainly of proteins and polysaccharides, is established. This layer is not thick enough to mask the hydrophobic and electrostatic forces from substratum despite the protein and carbohydrate layer. With any new material exposed to water, this conditioning film is the first step in establishing a biofilm on the surface. In addition to a conditioning film, the pipe material or substrate plays a large role in colonization of the surface by a biofilm. Different pipe materials exhibit different bacterial regrowth characteristics as shown by higher biofilm growth on iron pipe, followed by cement, and PVC (Camper, *et al.*, 2003).

Multiple techniques for quantifying and enumerating biofilm have been used. Measuring individual constituents of the biofilm such as carbohydrates, proteins, and phospholipids can serve to indicate the level and quantity of biofilm growth. Additionally, total biofilm amounts can be quantified by using well known tests such as total organic carbon, or chemical oxygen demand. Cells present can be quantified using culture methods to quantify the heterotrophs present. In addition to culture, genomic equivalents have been measured in biofilm samples using polymerase chain reaction to quantify the number of cells present in the samples (Lazarova & Manem, 1995). Drinking water biofilm quantitation is generally more challenging than that for non-drinking water biofilms due to the lower quantity of biomass available per surface area (Storey & Ashbolt, 2002).

Even though bacteria may adhere to a surface within a distribution system, a disinfectant residual still has the capacity to limit growth within the system. LeChevallier *et al.* point out that microbial surface growth (biofilm) within a system is unavoidable; the goal of the disinfectant residual is to control the growth, not eliminate it (LeChevallier *et al.*, 1988). In another study, bacterial levels after pigging initially decreased 10,000 fold, but that after just one week, average HPC counts return to pre-flush levels illustrating the ability of biofim to re-colonize surfaces of distribution systems.

As would be expected, thicker biofilms are typically found in systems with lower shear rates, and disinfection residuals, whereas, thinner biofilms are found in systems with higher shear rates and higher disinfection residuals. In high shear conditions, the biofilm bacteria are required to overcome the shear stress at the boundary layer which makes the film thinner and more resilient (Rickard *et al.*, 2004). Disinfection residual, in addition to shear, impacts the level of biofilm growth in a system. However, biofilm is still able to grow despite the presence of chlorine. It has shown that there is a distinct chlorine gradient over distances within a few hundred micrometers or less of a surface. Using chlorine microelectrode, it has been shown that the chlorine levels right at the surface of the biofilm were typically 20-30% of the bulk concentration. Therefore, the ineffectiveness of chlorine to oxidize biofilm is mainly due to limited penetration from a reaction - diffusion interaction (De Beer *et al.*, 1994). De Beer *et al.* did, however, find that biofilms are not homogeneous. Voids or channels in the biofilms may act as transport channels through which chlorine can penetrate in to the biofilm. Highly resistant spots of biofilm that are not penetrated may be caused by a higher cell density, or a higher density

of extracellular substances. Furthermore, the boundary layer thickness was observed to be negatively correlated to the liquid velocity. This finding is important in light of decontaminating a water system when chlorine is used. It seems that flushing at high velocities with higher amounts of chlorine would enable the chlorine to penetrate into the biofilms. With respect to spores attached to biofilm, Szabo *et al.* found that corroding iron was able to harbor and hold pathogens such as spores, even with higher levels of chlorine, creating a protective area for the spores to attach to (Szabo, *et al.*, 2007). This layer of viable cells coated with polysaccharides and excreted cellular products (Sutherland, 2001a) adds a level of complexity to predicting the level of contamination that may occur in a distribution system. The biofilm matrix protects the community of bacteria and enhances its ability to remain viable and attached to the surface despite the presence of disinfectants and shear from water movement along the pipe wall. Its presence may also increase or decrease the affinity for bacteria to adhere. Distribution systems contain different amounts of nutrients as well as different hydraulic characteristics which will influence the level of biofilm growth. Hydraulic conditions and pipe materials may make the distribution of biofilm irregular, making it difficult to define a "standard" drinking water biofilm. Despite the different biofilm levels in drinking water systems, Ollos *et al.* stated that a bacterial cell count of 10^4 CFU/cm^2 or higher is considered to adequately represent a viable drinking water biofilm (Ollos, *et al.*, 2003).

1.5 Artificial Biofilm

Attempts have been made to produce aqueous gels as artificial biofilm to be used for research purposes. The objective in producing artificial biofilms is to create well

characterized representative surfaces that mimic properties of biofilm without the need to grow living biofilms. Growing natural biofilm requires time (usually over 1 month) and requires specialized equipment and facilities. Additionally, living biofilm consists of live cells, polysaccharides, proteins, and other cell materials such as DNA which are different for each system and may differ within a distribution system. Working with artificial biofilm enables researchers to quickly produce films that have many of the same characteristics of the real world biofilms. Approaches to producing artificial biofilms have consisted of using purified polysaccharides such as alginate or agarose to form "hydrogels" which has been defined by Wingender *et al.* (1999b) as "polymeric, three dimensional networks which are swollen with a large excess of solvent." An examples of research involving artificial biofilm include studies evaluating the resistance of bacteria immobilized in the biofilm to disinfectants (Chen & Stewart, 1996). Other research has involved harvesting alginate from *Pseudomonas aeruginosa,* where it was sterilized, placed on plates, and then seeded with the live *P. aeruginosa* cultures. Oxygen profiles were then measured throughout the biofilm to measure kinetic parameters associated with respiration of the *P. aeruginosa* colonies (Abrahamson *et al.*, 1996). Living biofilms are generally physically and chemically non-homogenous natural gels consisting of the large molecules bound together by cross-linking and entanglement of the chains of polysaccharide including proteins, lipids, and other larger molecules(Wingender *et al.*, 1999) (Wingender, *et al.*, 1999). While these natural systems are quite complex, biopolymer gels such as gelatin, agarose, and calcium alginate produce similar structures to biofilm when hydrated. These polysaccharides have been the most convenient materials with which to produce simple models for artificial biofilm. One drawback to

using polyolysaccharide hydrogels is that the crosslinking of polysaccharides within the gel matrix allow molecules to diffuse into the matrix, however, the gel surface may be a barrier to particles and molecules which are larger than pore size (Rasmussen & Østgaard, 2003). This might not necessarilary be the case with living biofilm.

One procedure for producing artificial biofilm involves forming agarose beads that can then be placed or stuck to a surface. Porous agarose beads were originally developed for use in chromatography where larger pore spaces in the gel improved mass transfer to increase performance of the chromatographic separation (Gustavsson & Larsson, 1996). This agarose bead procedure was adapted by Strathmann *et al.* to more accurately mimic the properties of a real world biofilm expolysaccharides (Strathmann *et al.*, 2000). Strathmann developed porous (260 µm in diameter) and non-porous (50-500 µm in diameter) versions of the agarose beads. The pores in porous beads are on average 28 µm in diameter. The ability to produce porous structures in agarose shows promise since natural biofilm, as seen through the microscope, is porous, contains fissures, and interstices. Strathmann placed the agarose beads on surfaces to form films that were then compared with natural expolysaccharides to determine the differences and similarities in parameters such protective effects against oxidants as well as the water binding/retaining capabilities. Another aspect to the agarose bead procedure was that other compounds that are typically found in biofilm such as cells and proteins could be immobilized in the beads to more actively represent a natural biofilm.

1.6 Sampling

Once a contaminant is retained on the pipe surface, accurately measuring and knowing the level of contamination becomes the question. Accurately recovering bacteria off of dry surfaces became important after 9/11 and the anthrax mailings where thousands of swab, wipe, and vacuum HEPA filter sock surface samples were taken and sent to the lab for analysis. Swabs and wipes were used to sample non-porous surfaces such as painted wallboard and glass and the non-porous surfaces such as concrete and carpet were typically sampled with HEPA socks. One of the lessons learned was that information on sampling efficiency or the percent recovery off the surface was needed in order to interpret results. The realization was that it could not be assumed that negative sample results indicated the absence of *Bacillus* spores on the sampled surface. The compounded losses throughout the process of removal, extraction, and quantitation amounted to a proportion of spores that could not be accounted for with the particular method. This proportion is referred to as recovery efficiency or sampling efficiency and accounts for the compounded losses of removing the bacteria from the surface, extracting the bacteria from the sampling device, and then quantifying the bacteria in the resulting sample. Because of the lack of knowledge on recovery efficiencies for the various sampling techniques, subsequent testing was conducted to determine the recoveries that could be expected with each technique given different contaminated surfaces. To illustrate recovery efficiency, Brown *et al.* (Brown, *et al.*, 2007) contaminated two non-porous materials representing surfaces contaminated with aerosolized spores. The coupons were then sampled and processed using the (CDC)-recommended procedures for collection of *B. anthracis* spores. Recovery efficiency, which represented the effectiveness of transfer

from surface to plate counts, was calculated. It was found that recoveries for stainless steel and painted wallboard ranged between 25 and 40 percent recovery. Brown *et al.* also looked at the differences in recovery efficiency between low and high surface loadings and found that, at least with wipes, there was not a statistically significant difference between them. This study and others like it show the importance of knowing recovery efficiencies so that the true bacterial concentration on the surface can be more accurately estimated.

Surface characteristics considered relevant for dry surface sampling techniques (such as porous vs. non-porous surfaces) play an important role in sampling wetted internal drinking water distribution system components. Porosity and roughness of pipe material and biofilm along with the presence of these materials in the sample may inhibit the ability to detect the contaminant. Examples in the literature for pipe wall sampling include using a swabbing technique to remove biofilm from exhumed iron pipe to recover *Helicobacter pylori* (Mackay *et al.*, 1998). In another study, pipe samples were collected during their replacement and cut horizontally into two pieces so that the internal surfaces were accessible. Fluorescence in situ hybridization (FISH) was then used for direct detection of *Escherichia coli* on the pipe wall (Juhna *et al.*, 2007). In a study on bacterial regrowth in distribution systems, Camper *et al.* (2003) excavated 6 foot sections of pipe which were then capped and sent back to the laboratory. A 25 cm^2 template was placed over the pipe and biofilm and scale within the template area was scraped and placed in sterile water. The samples were spread plated on R2A agar to quantify the bacterial growth (Camper, *et al.*, 2003). Theses studies provide insight into sampling techniques

that can be used for pipe wall sampling; however, they do not provide information on recovery efficiency of the particular technique used.

In contrast to wetted pipe surfaces, the food industry uses swabbing with sponges, scraping, and excision sampling to quantify and verify the presence or absence of *Escherichia coli* and *Salmonella* on carcasses (Smith *et al.*, 2007). In addition to swabbing and wiping, scrape sampling with a blunt-edged stainless steel blade (Smith, *et al.*, 2007) and excision samples taken by cutting an areas out of the carcass with a cork boarer, blade, and forceps (Pearce & Bolton, 2005) have been used. In addition to these sampling techniques, rinsing the carcasses are standard practices in the food industry to detect and quantify bacterial contamination.

CHAPTER 2: Evaluation of surface sampling techniques for collection of *Bacillus* spores on common drinking water pipe materials

Material contained in this chapter has been formally published

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2.1 Summary

Drinking water utilities may face biological contamination of the distribution system from a natural incident or deliberate contamination. Determining the extent of contamination or the efficacy of decontamination is a challenge, because it may require sampling of the wetted surfaces of distribution infrastructure. This study evaluated two sampling techniques that utilities might use to sample exhumed pipe sections. Polyvinyl chloride (PVC), cement-lined ductile iron, and ductile iron pipe coupons (3 cm x 14 cm) cut from new water main piping were conditioned for three months in dechlorinated Cincinnati, Ohio tap water. Coupons were spiked with *Bacillus atrophaeus* subsp. *globigii*, a surrogate for *Bacillus anthracis.* Brushing and scraping were used to recover the inoculated spores from the coupons. Mean recoveries for all materials ranged from 37±30% to 43±20% for brushing vs. 24±10% to 51±29% for scraping. On cement-lined pipe, brushing yielded a significantly different recovery than scraping. No differences were seen between brushing and scraping the PVC and iron pipe coupons. Mean brushing and scraping recoveries from PVC coupons were more variable than mean recoveries

from cement-lined and iron coupons. Spore retention differed between pipe materials and the presence of established biofilm also had an impact. Conditioned PVC coupons (with established biofilm) had significantly lower spore retention (31±11%) than conditioned cement-lined coupons (61±14%) and conditioned iron coupons (71±8%).

2.2 Introduction

In the past decade, especially following the terrorist events of September 11, 2001 and subsequent anthrax mailings, the possibility that persistent biological agents might be used to disable critical infrastructure has received increasing attention. This paper examines the biological aspect of this scenario with a specific focus on bacterial endospores. Even in the presence of chlorine, bacterial endospores are persistent on internal pipe surfaces. It has been shown that they are able to persist for long periods even in the presence of chlorine following a contamination event(Morrow *et al.*, 2008; Szabo, *et al.*, 2007)[1,2]. It has also been shown that the presence of biofilm and corroding surfaces in drinking water distribution systems may influence adhesion of planktonic bacteria (Simoes, *et al.*, 2007).

There are currently protocols for collecting bulk water samples from a distribution system to detect microbial contamination. However, there are no standardized methods that include sampling of the internal surfaces of drinking water infrastructure for either intentional or unintentional contamination. The purpose of this study was to evaluate the precision and recovery efficiency of two different surface sampling techniques that could potentially be used to sample the internal surfaces of water distribution pipes

contaminated with bacterial endospores. Research on drinking water biofilms has generally used standard microbiological surface sampling techniques such as swabbing to recover biofilm from wetted pipe surfaces(Hallam *et al.*, 2001). However, to evaluate other surface sampling methods, brushing and scraping of three different types of pipe material were compared to determine which was the most effective in removing spores. Spore retention on different pipe materials was also evaluated.

2.3 Experimental

2.3.1 Pipe Material

PVC, unlined ductile iron, and cement-lined ductile iron were used in this study. These materials were chosen based on the results of a database search of the 2002 American Water Works Association (AWWA) survey of 337 small, medium-sized, and large utilities in the United States and Canada. The search showed that PVC, iron (including cast iron), and cement-lined pipes were found in United States' and Canada's distribution systems 8%, 15%, and 50% of the time, respectively[5]. Each of the pipe materials came from new 20.32 cm water mains obtained from IPEX (Mississauga, ON) (PVC) and American Ductile Iron Pipe Company (Birmingham, AL) (ductile iron and cement-lined ductile iron). Coupons (3 cm x 13 cm) were cut from 20.32 cm pipes using a high-pressure water jet cutter, which kept the coupon surfaces cool as they were cut. Each coupon provided a 39 cm^2 area to sample. Prior to use, coupons were washed and disinfected using the National Sanitation Foundation International (NSF) procedure for conditioning drinking water contact materials prior to determining the toxicity of the surface (ANSI/NSF, 2000). This involved scrubbing the coupons in tap water with a test

tube brush to remove any debris, spraying the coupons with 200 mg/L sodium hypochlorite solution coating all surfaces of the coupon, and then after 30 minutes, rinsing the coupons in deionized water. The coupons were then placed into the recirculation tank.

2.3.2 Recirculation Tank

A 1136-liter recirculation tank, with a residence time of approximately 14 hours, was used to expose the coupons to dechlorinated Cincinnati tap water. The average chlorine concentration of the incoming tap water was 0.99 mg/L with a standard deviation of 0.11 during the time period. A chemical feed pump delivered approximately five grams of sodium thiosulfate into the recirculation tank every 24 hours to achieve 0.01 to 0.05 mg/L of detectable free chlorine in the tank. The tank chlorine levels were measured weekly and the sodium thiosulfate feed rate was adjusted to keep the chlorine residual in the tank between 0.01 and 0.05 mg/L. The tank was gently mixed with a small electric mixer, which exposed the coupons to some shear forces.

Stainless steel racks were built to suspend each type of coupon material vertically in the tank. Each rack supported approximately 30 coupons stacked on top of each other and held in place with a stainless steel rod running through the middle of the rack. The curvature of the coupons made it possible for the rod to hold the coupons in place with contact only to the edges of the coupon. The rack and coupons are shown in Fig. 1.

Figure 1. Racks holding coupons in recirculation tank. Pipe material (from left to right) is PVC, cement-lined, and iron.

2.3.3 Biofilm Enumeration

Three coupons of each type of pipe material were sampled to collect biofilm for enumeration. Biofilm was brushed from the surfaces using adult soft toothbrushes (CVS Pharmacy, Inc., Woonsocket, RI, catalog no. 100982). Each coupon was brushed and subsequently washed a total of four times using a total of 50 mL volume of the phosphate buffer (Standard Methods Section 9216 B)(A.D. Eaton, 2005). Samples were diluted in phosphate buffer and cultured on R2A agar using the spread plate method and incubated at room temperature (21-24) for 7 days (Section 9215 C). Mean HPC counts for the biofilm developed on each material were: PVC, 1 x 10^5 CFU/cm^2, cement-lined, 4 x 10^5 CFU/cm^2, and iron, 3 x 10^8 CFU/cm^2. Simoes et al ((Simoes, *et al.*, 2007) found that biofilms grown in chlorine-free tap water on PVC under laminar conditions generated 10^4

CFU/cm^2. Also, according to a literature review conducted by Ollos *et al* (Ollos *et al.*, 1998), a bacterial cell count of 10^4 CFU/cm^2 or higher represents a viable drinking water biofilm.

2.3.4 Bacillus Spore Selection

A literature search was conducted to find a spore-forming *Bacillus* species that could be used as a surrogate for *B. anthracis.* One study showed that *Bacillus atrophaeus* subsp. *globigii* (BG) spores have a mean CT (C is the concentration of chlorine in mg/liter, and T is the exposure time in minutes) value higher than the mean CT values for *B. anthracis Sterne, B. cereus, and B. thuringiensis* along with the virulent Ames strain of *B. anthracis* (Sivaganesan *et al.*, 2006). Because of its increased resistance to chlorine, BG was selected since it represents a conservative surrogate for future drinking water pipe disinfection research.

2.3.5 Spore Preparation, Growth, and Enumeration

BG spores were obtained from the U.S. Army's Dugway Proving Ground (Dugway, Utah) and were grown according to the methods described in Nicholson and Setlow(Nicholson & Setlow, 1990) and Coroller, Leguerinel et al. (Coroller *et al.*, 2001). Generic spore media was inoculated with vegetative BG cells and incubated for five days at 35°C with gentle shaking in a rotary shaker. Purified BG endospores were produced using gradient separation as described by Nicholson and Setlow and the presence of spores was confirmed using phase contrast microscopy (<0.1% vegetative cells). Spores were stored in 40% ethanol at 4°C until use. Spores used in this study came from the same batch and container.

Spores were enumerated using serial dilution and the spread plate method (Standard Methods Section 9215 C) with 0.1 mL of the sample on tryptic soy agar (TSA). The diluent used was phosphate buffer (Standard Methods Section 9216 B) with 0.01% Tween® 80 (Fisher Scientific, Pittsburgh, PA, catalog no. AC278630000). Tween® 80 is a surfactant that has been shown to improve spore recovery (Brown, *et al.*, 2007). All samples, including quality control (QC) samples, were spread plated in triplicate with the exception of the tap water spore suspension, which was spread plated using 5 replicate plates to generate a more precise value. Spore samples from coupons were heat shocked at 80º C for 10 minutes to inactivate vegetative organisms. Samples were cooled and vortexed prior to plating. Plates were incubated for 24±2 hours at 35°C. Because it forms orange colonies, BG was easily distinguished from other biofilm organisms that survived the heat shock.

2.3.6 Tap Water Spore Suspensions

Fresh spore suspensions were made on the day of the experiment. Suspensions were made by adding 40 µL of the concentrated spore stock suspension, described above, to 50 mL of dechlorinated Cincinnati tap water resulting in a 6×10^5 CFU/mL suspension. Dechlorinated water from the tank was used as the diluent so that the recirculation tank water quality was represented.

2.3.7 Water Parameter Monitoring

Conductivity, temperature and pH of the recirculation tank water were measured at each coupon sampling event. Average values of 340 µS/cm (SD=190) for conductivity and 13.7 ºC (SD=0.015) for temperature were recorded using an Extech conductivity meter (Extech instruments, Waltham, MA, catalog no. 407303). pH ranged from 8.22-9.00 with a median value of 8.27 and was measured using an Orion 555A pH/ORP/Conductivity Meter (Thermo Fisher Scientific Inc., Waltham, MA). Total chlorine was measured twice a week using the Hach total chlorine method 8167(4500-Cl Standard Methods Chlorine DPD Colorimetric Method) using Hach DPD free chlorine reagent AccuVac® ampules (Hach, Loveland, CO, Catalog no. 25020-25), with a Nalco 2800 DR spectrophotometer (Nalco Company, Naperville, IL). Chlorine was found to be less than 0.05 mg/L in the tank due to the introduction of sodium thiosulfate. On four occasions, additional recirculation tank water samples were collected, heat shocked, and spread plated on TSA to determine whether spores were present in the bulk water in the tank.

2.3.8 Coupon Collection and Spore Loading

Individual coupons were removed from the coupon rack and placed into a sterile 1,000 mL beaker while still submerged in the tank. Each coupon was then placed in a sterile 14 cm diameter Petri dish and was leveled using a sterile level. A two step procedure was used to load the coupons with spores: the suspension on the coupons was spiked; then, the coupons were rinsed to remove spores that had not been retained on the surface.

Spore Spiking: After the coupon was leveled, it was spiked with 0.5 mL of dechlorinated tap water containing approximately 3 x 10^5 spores. The spore suspension on the coupon was then spread with a sterile 250 μm pipette tip to ensure that all parts of the coupon were exposed to the suspension. The suspension was left on the coupon for 20 minutes.

Coupon Rinsing: After 20 minutes, the contaminated surface of the coupon was manually rinsed with 100 mL of dechlorinated tap water. Dechlorinated tap water was pumped from a sterile 1 liter beaker using a peristaltic pump calibrated to pump approximately 120 mL per minute. The rinsing procedure was timed to ensure that 100 mL of water was used in the rinsing process. Spores rinsed off the coupon were collected in the same Petri dish that was used to hold the coupon while it was spiked with the spore suspension. This rinsing procedure removed any spores not retained (or weakly retained) on the coupon surface.

2.3.9 Coupon Sampling

Sampling techniques were compared on the three different pipe surfaces to determine differences in recovery and precision. These methods were chosen due to their low cost and availability, as well as their potential to remove biofilm and loose corrosion from porous and non-porous surfaces.

Toothbrush: A search was conducted to find low-cost, disposable brushes with bristles on one side that could be used to remove loose biofilm and scale from porous wetted surfaces. It was found that brushes designed for scientific uses, such as test tube brushes, were either too expensive or were designed with bristles all around the shaft. Soft adult

toothbrushes were the right design and relatively inexpensive (CVS Pharmacy, Inc., Woonsocket, RI, catalog no. 100982). These toothbrushes, available in bulk for less than a dollar per unit, were used for all of the brushing experiments.

Cell Scraper: The cell scraper was chosen because, like the toothbrush, it was disposable, easy to use, and relatively inexpensive when purchased in bulk at less than $2.50 a unit. Designed for use in harvesting cells from culture vessels, it was logical choice for removing corrosion, biofilm, and adhered contaminants from drinking water pipes. Sterile Fisherbrand cell scrapers (Fisher Scientific, Pittsburgh, PA, catalog no. 08-773-2) with plastic 1.8 cm blades were used for the scraping experiments.

Spore Sampling from Coupon Surface: The brushing and scraping procedure was the same for each coupon. Coupons were brushed with 10 downward strokes, ensuring that all of the material brushed from the coupon was collected in the Petri dish. After the 10 brush strokes, the coupon and brush were washed with phosphate buffer and 0.01% Tween® 80 contained in a sterile 150 mL squirt bottle. The scraping procedure consisted of scraping the coupon from both directions horizontally so that the biofilm/spores were scraped to the center of the coupon. After the entire surface of the coupon was scraped, it was washed with phosphate buffer and 0.01% Tween® 80 in the same manner that was done with the brushing samples.

Each coupon was brushed or scraped, then washed, four times. A combined 50 mL of the phosphate buffer and 0.01% Tween® 80 was used in the process, resulting in a 50 mL

sample that was collected in the Petri dish. This suspension was transferred to a 100 mL sample container. The quantity of spores found in this sample was compared to the number of spores that were calculated to be on the surface prior to sampling, resulting in a recovery efficiency value.

2.3.10 Other Sampling and Quality Control Checks

Additional spore sampling was conducted to determine where losses may have occurred throughout the sampling and analysis process. Quality control checks were also routinely done to check for potential contamination issues.

Sides and Back Check: To determine the extent of contamination on the backs and sides of the coupons throughout sampling, the sides and back of each coupon were brushed using 50 mL of phosphate buffer with 0.01% Tween® 80 as a diluent to rinse the brush and coupon during and after brushing. This procedure for sampling the sides and back of each coupon was the same as the brushing procedure described in the previous section. The resulting spore suspension was collected in a sterile Petri dish and transferred to a 100 mL sample container.

Petri Dish Check: In order to determine the extent of spore retention on the walls and bottom of the Petri dishes used to collect samples, the dishes were swabbed using sterile macrofoam swabs (ITW Texwipe® CleanTip® Swabs, Fisher Scientific, Pittsburgh, PA, catalog no. 18-359) after transferring the samples to 100 mL sample containers. Each swab was placed in a sterile 50 mL test tube containing 10 mL of phosphate buffer and 0.01% Tween® 80. The tube was then vortexed for 2 minutes using 10-second intervals

to remove the spores from the swabs. Swabs were then rolled on the inside of the tubes to remove as much liquid from the swab as possible and the swabs were discarded. Tubes containing spore suspensions were then heat shocked and 0.1 mL of the suspension was then spread plated on TSA to enumerate spores.

Brush and Scraper Check: Spore losses due to retention on brushes and scrapers were checked after the brushes and scrapers were used to sample the coupons. Each brush or scraper used in the experiments was placed in a sterile 50 mL test tube containing 10 mL of phosphate buffer and 0.01% Tween® 80 and was vortexed as described above for the swabs. Spores removed from the brushes and scrapers were enumerated using the same procedure as was used with the swabs.

Matrix Effect Check: Corrosion and/or biofilm collected from one conditioned coupon of each pipe material were used to determine the impact of the matrix on spore recovery. 6×10^4 CFU were added directly to 100 mL of phosphate buffer containing the scraped matrix and 0.01% Tween® 80. Undiluted samples from these treatments and a phosphate buffer/Tween® 80 control were enumerated for BG spores and recoveries were calculated.

Conditioning Control Coupons: To determine the effect of the 3 month conditioning process had on spore retention, 12 additional coupons (4 each of PVC, unlined ductile iron, and cement-lined ductile iron) were washed and disinfected using the NSF International procedure described in the pipe material section. The coupons were then

placed in 3 Liter buckets of autoclave sterilized Cincinnati tap water for 24 hours to hydrate the surfaces. These coupons were then spiked with spores and rinsed using the same procedure described in the proceeding section.

Contamination Checks: To ensure that TSA, R2A plates were sterile, blank plates were incubated along with samples during every sampling event. Phosphate buffer used in the experiments was spread plated on TSA to determine sterility. On four occasions, coupons of each pipe material were collected from the recirculation tank to take HPC counts as described in the biofilm enumeration section. In addition to biofilm enumeration, the biofilm/corrosion collected from the coupons was heat shocked and the resulting sample was spread plated on TSA to verify that the coupons were not contaminated with BG spores prior to experimentation. Fresh spore suspensions were made from the same stock suspension each time coupons were spiked. Each suspension made was spread plated to check spore viability and to enumerate the number of spores per unit volume. The mean spore concentration for all of the spore suspensions made was $3.1 \text{ X } 10^5$ with a standard deviation of $4.8 \text{ X } 10^5$. Additionally, prior to conducting sampling experiments, biofilm and corrosion from one coupon of each pipe material was collected, spiked with BG spores, and enumerated to determine the matrix effect from the corrosion and biofilm.

2.3.11 Mass Balance Calculation

A mass balance approach was used to calculate the recovery efficiency of the sampling methods. The specific components (Spike C_o, rinse C_r, brush or scrape C_s, and sides and back C_b) are summarized in Table 1.

Table 1. Samples from spore suspensions, coupons, and sampling equipment used in mass balance calculation*

	Samples	Description	Reason for sample
Coupon Inoculation spore suspension **(C_o)**	Sample	Tap water spore suspensions used to inoculate coupons were quantified to determine the initial number of spores inoculated onto coupon	To quantify the total number of spores that were spiked onto the coupon
Rinse **(C_r)**	Sample	Following inoculation, coupons were rinsed with 100 mL of dechlorinated tap water to remove spores that have not been retained on the surface.	To quantify the number of spores that were rinsed off of the coupon with dechlorinated tap water
	Petri dish check	Determination of the number of spores that stay attached to the Petri dish	To quantify spores retained to Petri dish from rinse sample
Brush or Scrape **(C_s)**	Sample	Following rinsing, the side of the coupon corresponding to the internal pipe surface was brushed or scraped	To quantify spores that were brushed or scraped from the coupon.
	Toothbrush or scraper check	Determination of the number of spores that stay attached to the toothbrushes and scrapers	To quantify spores on toothbrush or scraper
	Petri dish check	Determination of the number of spores that stay attached to the Petri dish	To quantify spores retained to Petri dish from toothbrush or scraper sample
Sides and Back **(C_b)**	Sample	Following brushing or scraping, the sides and back of each coupon were brushed.	To quantify the number of spores found on the sides and back of each coupon
	Toothbrush check	Determination of the number of spores that stay attached to the toothbrushes and scrapers	To quantify spores on toothbrush
	Petri dish check	Determination of the number of spores that stay attached to the Petri dish	To quantify spores retained on Petri dishes from sides and back sample

*All samples are spread plated in triplicate

Spore recovery efficiency (RE) was computed as shown in Equation [1] using the terms defined in Table 1. Percent retention (PR) of spores was computed as shown in Equation [2].

$$RE = \frac{C_s}{C_o - (C_r + C_b)} \times 100 \qquad [1]$$

$$PR = \frac{C_o - C_r}{C_o} \times 100 \qquad [2]$$

2.3.12 Experimental Design

Seventy two coupons including quality control coupons were used in the experiments. Twelve of the 72 coupons were conditioning control coupons described above. An additional twelve of the recirculation tank conditioned coupons were sacrificed in order to enumerate biofilm and conduct quality control checks. The remaining 56 coupons were used for the sampling experiments as shown in Table 2.

Table 2. Experimental design for pipe coupons

	Conditioning control coupons (no biofilm)	Recirculation tank conditioned coupons. (established biofilm)			
		Biofilm and procedural blank samples	Brushing	Scraping	TOTAL
PVC	4	4	8 (7)	8 (7)	24 (18)
Cement-Lined	4	4	8 (8)	8 (7)	24 (19)
Iron	4	4	8 (8)	8 (7)	24 (19)
					72 (56)

Numbers in parentheses are actual numbers of coupons used in recovery efficiency and percent retention experiments

2.3.13 Statistical Analysis and Calculations

The experiment was designed for analysis using a 2-way analysis of variance (ANOVA). However, spore recovery efficiency (RE) data were not equally variable among pipe materials (see data in Fig. 2), violating one of the underlying assumptions for an ANOVA analysis. Therefore, recovery efficiency data were analyzed with t-tests using SigmaPlot® software (SYSTAT Software, Inc., San Jose, CA) with a 0.05 significance level. The experimental design enabled testing of the null hypothesis that assumed there were no differences in recovery efficiencies between the sampling techniques for each pipe material. Percent retention (Equation [2]) was tested using a one-way analysis of

variance. The null hypothesis for this design was the assumption that there would be no significant differences in retention among pipe materials.

2.4 Results

2.4.1 Recovery Efficiency

Recovery efficiency values were calculated using Equation 1 and are shown in Table 3 and Fig. 2. Data were then arcsin squareroot transformed prior to conducting statistical tests. Mean recovery efficiency values for brushing and scraping for combined pipe materials were 0.41 (sd=0.20) for brushing, and 0.40 (sd= 0.21) for scraping. Combined recovery efficiency for brushing and scraping on all pipe materials was 0.40 (sd=0.20). T-test results showed significant differences between cement-lined brushing and scraping (P=0.003). There were no significant differences between brushing and scraping for PVC (P=0.092) and iron (P=0.691).

Table 3. Mean, median, and standard deviation values for recovery efficiency of *B. globigii* spores from conditioned PVC, cement-lined, and iron pipe coupons.

	PVC			Cement			Iron	
	Brush	Scrape		Brush	Scrape		Brush	Scrape
Sample Size	7	7		8	7		8	6
Mean	0.37	0.51		0.42	0.24		0.43	0.46
Median	0.29	0.47		0.42	0.21		0.44	0.46
SD	0.30	0.29		0.09	0.10		0.20	0.04

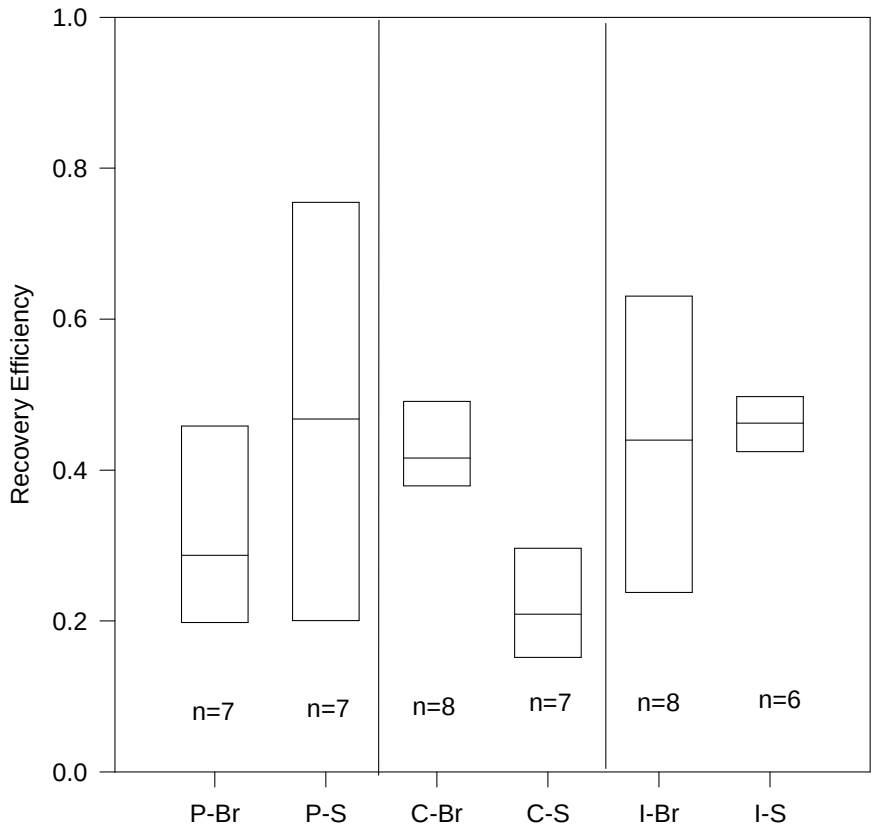


Figure 2. Recovery of *B. globigii* spores from PVC (P), cement-lined (C), and iron (I) pipe coupons using brushing (Br) and scraping (S). The line in the middle of the box indicates the median and the ends of the boxes define the 25th and 75th percentiles. Whisker bars are not shown due to sample size ≤ n=8.

2.4.2 Percent Retention

Percent retention of *B. globigii* was calculated using Equation [2]. Fig. 3 illustrates the differences in retention between the various pipe materials.

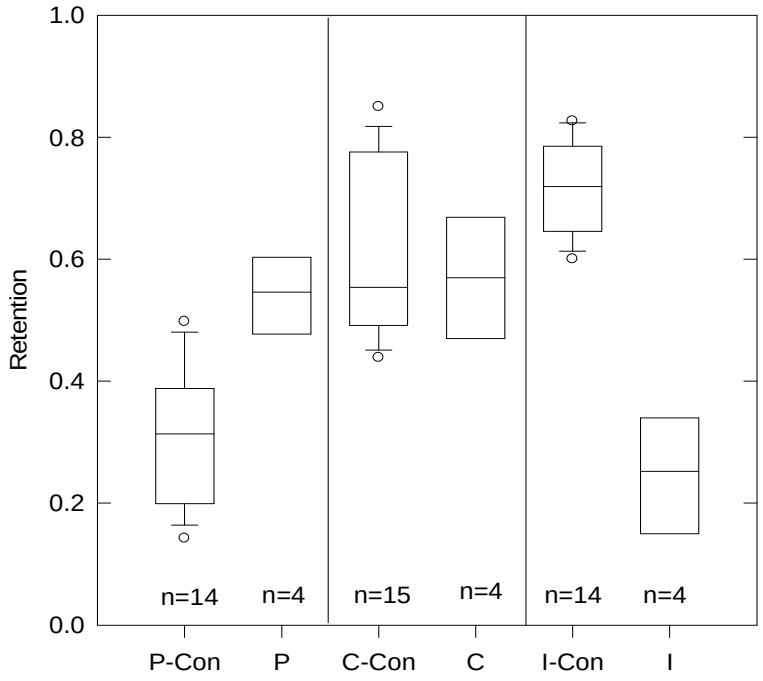


Figure 3. Comparison of B. *globigii* retention on PVC (P), cement-lined (C), and iron (I) conditioned (Con) and unconditioned pipe coupons. The line in the middle of the box indicates the median and the ends of the boxes define the 25th and 75th percentiles. Whisker bars define the 10th and 90th percentiles.

There were significant differences between PVC and cement-lined and PVC and iron coupons that had been conditioned. In contrast, no significant differences between conditioned cement-lined and iron pipe coupons were found. Additionally, significant differences were seen between conditioned and unconditioned PVC, and conditioned and unconditioned iron pipe material.

2.4.3 Sides and Back

As part of the mass balance calculation, the sides and backs of all coupons were brushed and rinsed to determine the number of spores that may have migrated there during the spiking procedure or during the rinsing, or the brushing or scraping process. It was observed that spore suspensions on the PVC coupons pooled up on the surface, whereas

the cement-lined pipe coupons either absorbed or wicked the suspension off the surface during the 20 minute contact time. Data generated from analyses of the cement-lined pipe coupon sides and back show that 11.8% of the inoculated spores were recovered there. In contrast, 0.14% and 0.10% of the spores were recovered from the sides and back of the iron and PVC, respectively.

2.4.4 Relative Proportions of Spores in Each Sampling Step

Mean spore recovery for each of the steps is summarized in Fig. 4. The height of the bar represents the mean number of spores spiked onto the coupon. Sections within the bar represent the relative proportions of spores that were recovered in each of the sampling steps described in Table 1.

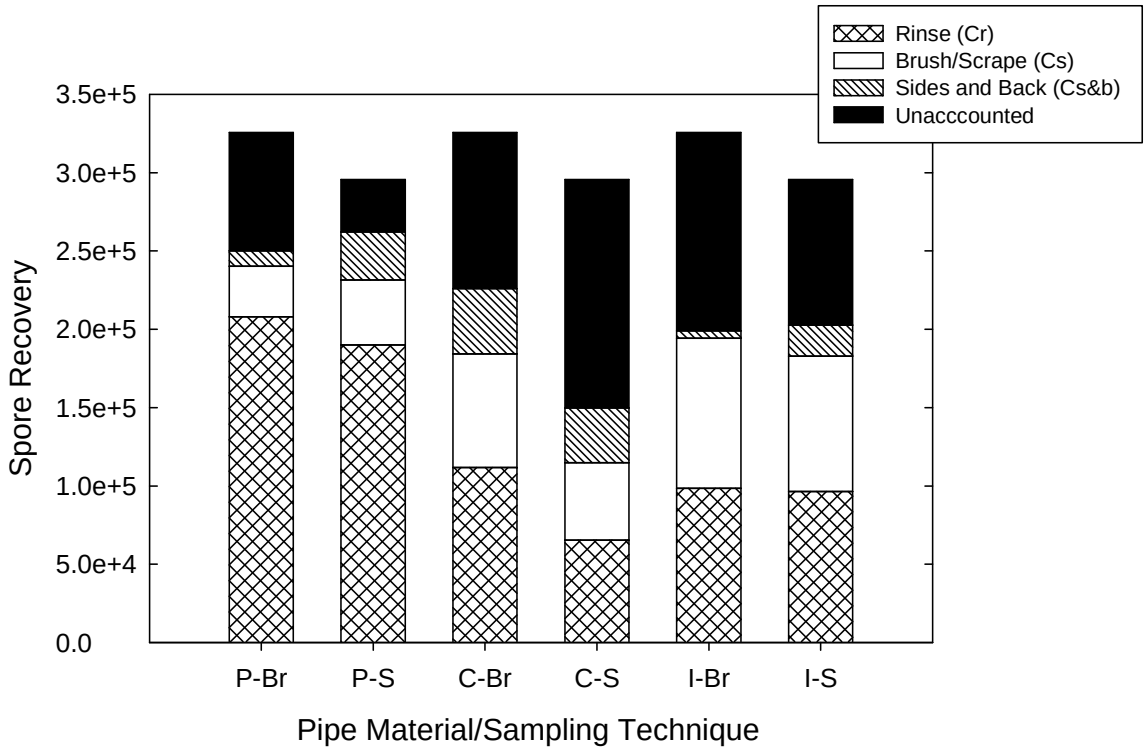


Figure 4. Mean spore recovery for all techniques. Each bar represents the mean number of spores spiked on the coupon (8 brushed (Br) and 7 scraped (S) coupons. Relative numbers of spores recovered are shown as mean values for C_r, C_s, and C_b are shown in relation to the number of spores spiked on PVC (P), cement-lined (C), and iron (I) coupons

Spore Losses to Petri Dishes, Tooth Brushes, and Cell Scrapers

Spore losses to retention on Petri dishes, toothbrushes, and scrapers, are shown in Table 4. Percentages indicate the number of spores recovered with respect to the total number spiked on the coupons.

Table 4. Mean counts of spores sampled from Petri dishes, toothbrushes, and scrapers

	Rinse Petri dishes (no Tween®)	Scrape Petri dishes	Sides and back Petri dishes	Toothbrushes	Scrapers
Avg. No. spores recovered (SD)	13557 (10172)	4078 (4851)	3423 (5651)	515 (482)	568 (912)
Percentage of spores recovered	4.30%	1.30%	1.10%	0.20%	0.20%

2.4.5 Impact of Tween® 80 and Corrosion on Spore Recovery

In preliminary studies, it was determined that there were significant losses due to spores clumping and adhesion to sample containers. Thus, 0.01% Tween® 80, was included in all phosphate buffer used to collect and enumerate spores. The purpose of the spore spiking and coupon rinsing steps was to simulate contaminated tap water and subsequent water movement in a pipe. Therefore, out of necessity, the spore spiking and rinsing steps did not include Tween® 80. To confirm the effect that adding a surfactant has on BG spore recovery, a phosphate buffer spore suspension was split into two identical samples and Tween® 80 was added to one of the samples. The suspensions were then spread plated on TSA. Approximately two times the number of spores were recovered in the presence of Tween® 80 (3153 per mL with vs. 1603 spores per mL without).

In contrast to the effects of Tween® 80 on spore recovery, iron corrosion was shown to have an adverse effect. Corrosion and/or biofilm was collected from uncontaminated but conditioned pipe coupons using the same procedure used for sampling coupons via brushing without Tween® 80. Then an aliquot from the same spore stock suspension was spiked into each sample to determine spore recovery in the presence of the corrosion and/or biofilm. Fig. 5 shows that there is a considerable difference between spore

recovery in the presence of iron corrosion and recovery in the presence of biofilm alone brushed from the PVC and Cement-lined pipe coupons. Furthermore, if samples containing iron corrosion were diluted by half phosphate buffer solution (and, later Tween® 80), spores were able to grow more readily on TSA. The dilution and plating protocol for the iron coupons was adjusted accordingly for the reported experiments.

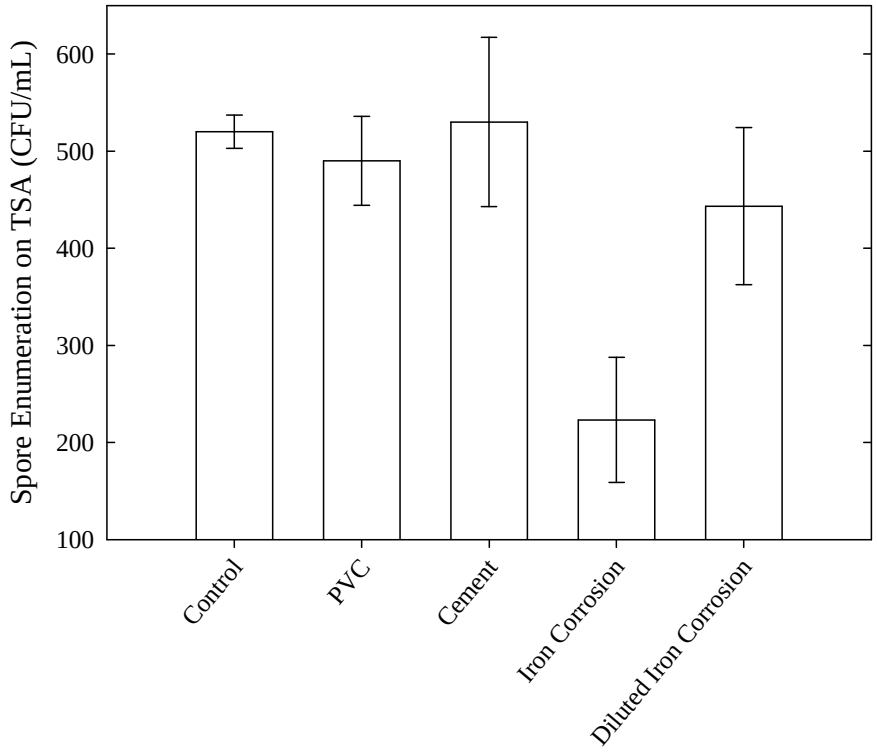


Table 5. Spore enumeration in the presence of biofilm and corrosion (3 plates per sample). Error bars represent standard deviations.

2.5 Discussion

This study examined two ways to sample attached bacterial spores from wetted porous and non-porous surfaces. When the mean recovery for all the brushing data is compared

with the mean recovery for all the scraping data (for all three pipe materials), it is clear that one sampling technique did not stand out as being more effective than the other. Brushing vs. scraping recovery efficiency data for all three pipe materials combined showed that one sampling technique was not more effective than the other. However, brushing cement-lined pipe coupons was shown to be more effective than scraping cement-lined pipe coupons. Results for recovery efficiency also differed in variability among the three pipe materials, 0.29, for PVC vs. 0.10, and 0.12 for cement lined and iron, respectively. This suggests that pipe material influences the variability of the recovery efficiency. Recovery efficiency standard deviations for iron coupons varied for brushing and scraping (0.22 vs. 0.04) while PVC (0.30 vs. 0.29) and cement-lined (0.09 vs. 0.10) coupons had approximately the same standard deviation for brushing and scraping, respectively. This difference between brushing and scraping observed for the iron coupons could be attributed to the layer of rust that absorbed and stabilized the spore suspension. It was noted that scraping removed the majority of the biofilm and rust layer with one stroke. In contrast, brushing required 3-4 strokes to remove the same amount of corrosion. Although there were no statistically significant differences between the mean recovery efficiencies for brushing and scraping on iron, variability in the data indicate that scraping may be the more desirable technique due to the higher level of precision.

One of the limitations in this study is that the corroded iron coupons may not be representative of tuberculated iron pipes found in real world distribution systems. Based on research involving characterization of iron pipe surface deposits found in pilot scale and real distribution systems, the layer of tuberculation would likely be harder to remove

than the iron corrosion formed on the iron coupons used in this study. Thus, for exhumed tuberculated iron pipe, a better surface sampling strategy might be to brush the outer layer of pipe scale to collect the soft corrosion and biofilm present on the surface. If spores are able to penetrate past the outer layer of corrosion, removing deeper layers of corrosion using a heavier chisel, scalpel, or spatula may be required to remove the entire deposit (Brown, Betty, Brockmann, Lucero, Souza, Walsh, Boucher, Tezak, Wilson, Rudolph, *et al.*, 2007). Due to the large amounts of iron corrosion that would be present in these samples spore growth would most likely be inhibited. Therefore, understanding the impact of corrosion on spore recovery from exhumed pipe should be verified through the use of positive controls, matrix spikes, and standard additions.

Recovery results for the cement-lined pipe coupons showed similar brushing and scraping variability. The significant differences between mean recovery efficiency of brushing and scraping were expected (42% versus 24%, respectively) due to the porous cement-lined surface. The toothbrush was likely more effective because it removed the spores that may have been stuck in the interstitial spaces and could not be accessed by the scraper. Therefore, for hard porous surfaces, brushing appears to be a more effective sampling technique that will produce the highest recoveries.

Recovery efficiency variability was highest for PVC pipe coupons for both brushing and scraping. Additionally, conditioned PVC had a lower level of spore retention than cement-lined or iron. This was expected for two reasons. First, PVC had lower HPC (less biofilm) than the cement-lined coupons. Second, the PVC surface was notably smoother,

providing less opportunity for spore retention. Iron pipe coupons had a higher HPC ($3x10^8$ CFU/cm^2). The large amount of corrosion and biofilm on the iron coupons may have provided additional surface area for immobilizing the spores within the matrix.

The 24 hour conditioned iron coupons did not have the opportunity to develop the same amount of corrosion and surface roughness as did the coupons conditioned for three months, and, not surprisingly, they did not retain spores as well as the iron coupons with more rust. PVC conditioning had the opposite effect. Spores were retained more readily on coupons with minimal conditioning than they were on the conditioned coupons with 3 months of biofilm growth (1×10^5 CFU/cm^2 for PVC). This may have been due to the physiochemical properties of the material such as charge or hydrophobicity of the PVC. Spores are generally hydrophobic and negatively charged which may account for a stronger attraction to the unconditioned PVC (Lytle, *et al.*, 2002; Tauveron *et al.*, 2006). Also, while not directly observed, biofilm sloughing may have occurred more readily from the smooth, hydrophobic surface of the PVC than from the cement-lined or iron coupons, taking the spores with it during the rinse.

The unclosed mass balances (Fig. 4) could be attributed to a number of different causes in addition to sampling inefficiency. First, spores collected during coupon rinsing (C_r) were exposed to tap water without Tween® 80. As seen by increased Petri dish swab counts for the rinse samples, a proportion of the spores that were not recovered probably had been retained on the sample containers and Petri dishes. This illustrates the importance of using a surfactant such as Tween® 80 in every step to increase recovery. The second loss

may be attributed to incomplete recovery of spores from the sides and backs of the coupons. Recovery efficiency of spores from of the sides and backs was not quantifiable. Thus, the proportion of the spores that were actually removed and enumerated may not have been representative of the true number of spores present on the sides and backs.

The reported data illustrate the potential for spore retention on different wetted pipe materials and associated sampling recovery from those materials. Since spore losses can be encountered throughout the sampling and analysis process, it is important to consider the recovery efficiency of the method used. Furthermore, in real-world situations, very low concentrations of surface contamination may be present making high recovery efficiency and precision of recovery all the more important. In selecting sampling techniques, this study shows that the pipe material characteristics and the presence of biofilm will influence recovery efficiency and recovery precision. Accordingly, the sampling technique should be selected based on its ability to remove spores from the particular surface with an acceptable level of precision. Brushing vs. scraping cement-lined coupons illustrates the differences in recovery efficiency that may be obtained for the same surface. Bacterial retention and bacterial sample collection from wetted surfaces is complex. Future work in this area should evaluate additional surface sampling methods for wetted pipe surfaces and methods for quantifying pathogens in the presence of pipe corrosion.

CHAPTER 3: *Bacillus* spore retention on common drinking water pipe materials

This chapter is a manuscript submitted for internal U.S. EPA review prior to submission to Applied and Environmental Microbiology.

3.1 Summary

Intentional or unintentional biological contamination is a potential problem in drinking water distribution systems due to the possibility of pathogens adhering to internal surfaces. If the bacteria remain viable in the system, over time they can detach from the internal surfaces in quantities above the infectious dose. This study evaluated bacterial endospore retention on new polyvinyl chloride (PVC) cement-lined ductile iron, and ductile iron, material commonly found in drinking water distribution systems. To determine the impacts of conditioning and presence/absence of biofilm, each material was prepared in three different ways – bare (new), conditioned (exposed to flowing city tap water for 13 months), and coated with artificial "biofilm" (agarose). A suspension of $2x10^4$ spores in 2 mL of dechlorinated tap water was applied to the coupon surfaces for 20 minutes followed by rinsing with dechlorinated tap water to collect spores to determine the number of spores released from the surface, and, by difference, the spore retention. Results were analyzed by directly comparing measured membrane filter plate count numbers for the numbers of spores released. A second study was conducted to

evaluate spore retention on artificial “biofilm” vs. a natural, but nutrient-augmented, biofilm grown in a recirculation tank. The second study was done at three different spore concentrations. Due to non-normal distributions, all data were analyzed using non-parametric statistics. Conditioned ductile iron retained significantly more spores than the other pipe materials as seen by the significant differences between conditioned PVC, conditioned cement, bare iron, and agarose-coated bare iron. Significant differences were also seen between conditioned cement and conditioned PVC. Tests comparing spore retention between agarose “biofilm” and a nutrient-augmented biofilm also showed that for all three concentrations, augmented biofilm significantly retained more spores than agarose “biofilm”. Augmented “natural” biofilm on PVC coupons retained more spores than agarose “biofilm” on PVC. In contrast, augmented “natural” biofilm on cement-lined coupons compared to agarose “biofilm” on cement-lined coupons showed no significant differences, leading us to believe that the agarose “biofilm” simulates spore retention as long as the natural biofilm is a certain thickness. Iron coupons with agarose “biofilm” applied also retained significantly more spores than PVC and cement-lined coupons leading us to believe that iron corrosion associated within the agarose matrix may enhance spore retention.

3.2 Introduction

The potential for intentional or unintentional contamination of water infrastructure with a persistent pathogen is a concern for water distribution managers. Bacterial endospores have been found, but are typically not looked for in drinking water distribution systems. They have been shown to be persistent in water even in the presence of chlorine (Watson

& Keir, 1994), (Maginnis, *et al.*, 2001). They have also been shown to attach to internal surfaces of water infrastructure including biofilm and corrosion (Szabo, *et al.*, 2007). Due to the impact contamination would have, it is important to evaluate and compare the contamination potential of various distribution system internal surfaces so that levels of contamination can be estimated. To address this need, this study evaluated bacterial endospore retention on three different pipe materials with three different types of surface preparations.

Bacterial retention on wetted surfaces is a complex phenomenon which is influenced by multiple factors. For planktonic bacteria such as a spore suspended in a drinking water pipe, water parameters such as pH and ionic strength impact the bacteria's affinity to adhere to surfaces from the water phase. This can be shown by differences in electrophoretic mobility (Lytle, *et al.*, 2002) and hydrophobicity (Faille, *et al.*, 2002) at different ionic strengths (Van Der Kooij *et al.*, 1995). Presence or absence of biofilm on the internal system pipe and components may increase or decrease spore retention within the system (Costerton *et al.*, 1994). Material or substrate properties include surface roughness (Riedewald, 2006), hydrophobicity, and surface charge of the material. Additionally, different materials tend to exhibit different biofilm growth potentials based on their material characteristics (Camper, *et al.*, 2003). Water parameters such as disinfectant residual (Chu *et al.*, 2003) and presence of organic matter in the water (Gagnon *et al.*, 1998) directly impact the level of conditioning films (Schneider, 1997) and biofilm that can form on internal surfaces. After a conditioning film has formed, biofilm in drinking water systems exist as a matrix of bacterial and other microorganisms

suspended in extracellular polymeric substances (hydrogels) that are 95-98% water. These hydrogels are composed of chains of polysaccharides, proteins, and may also contain lipids, glycolipids, and nucleic acids. These physically and chemically non-homogenous natural gels consist of the large molecules bound together by cross-linking and entanglement of the chains of polysaccharide including proteins, lipids, and other larger molecules (Sutherland, 2001a; Wingender, *et al.*, 1999). Naturally occurring biofilms have microcolonies encapsulated by the expolysaccharides with interstitial voids or channels within the biofilm (Costerton, *et al.*, 1994; Wingender, *et al.*, 1999). In contrast to naturally occurring biofilm which gels due to a number of different forces, classic biopolymer gels such as agarose, gelatin, or alginate consist of networks of crosslinks bonded together (Abrahamson, *et al.*, 1996). To illustrate, alginate is held together by cations and the agarose cross links consisting of non covalent bonds make the gel homogenous and stable for long periods of time. Because of the stability and ease of use, artificial biofilms using biopolymer gels have been used to simulate natural biofilms (Smetana Jr, 1993).One procedure for producing artificial biofilm involves forming semi-porous agarose beads which can be fixed to a surface to simulate a biofilm. This bead procedure, originally developed for use in chromatography to enhance chromatographic separation, improved mass transfer (Gustavsson & Larsson, 1996) and was adapted by (Strathmann *et al.*, 2000) to simulate the microchannels and structures that can be found in naturally occurring biofilms. Spherical beads were attached to surfaces to form films which more accurately mimic the properties of a real world biofilm expolysaccharides (Strathmann, *et al.*, 2000). In this procedure, porous beads approximately 260 µm in diameter with an average pore diameter of 28 µm were fixed in place using a dilute

agarose solution. Since having a convenient biofilm surrogate which is representative of a worst case scenario (due to thickness) may prove advantageous to evaluating adhesion of contaminants to drinking water biofilm as well as decontamination procedures, the superporous agarose bead procedure developed by Strathmann *et al.* was used in to produce artificial biofilm for evaluation in this study.

3.3 Materials and Methods

Pipe coupons (3 cm x 13 cm) pipe were cut from 10 inch water mains obtained from IPEX (Mississauga, ON, for PVC) and American Ductile Iron Pipe Company (Birmingham, AL, for ductile iron and cement-lined ductile iron) using a high-pressure water jet cutter as described in (B. H. Packard & Kupferle, 2010) Following cutting, the sides and backs of coupons were sealed with NSP 120 NSF/ANSI certified high performance epoxy (NSP Specialty Products, Pinehurst, NC). Epoxy was also formed around the periphery of the face side of all pipe coupons so that it would retain liquid as shown in Fig. 5. Prior to use, all coupons were washed and disinfected using the National Sanitation Foundation International (NSF) procedure for conditioning drinking water contact materials prior to determining the toxicity of the surface((ANSI)). This involved scrubbing the coupons in tap water with a test tube brush, spraying the coupons with 200 mg/L sodium hypochlorite solution to coat all surfaces, and then after 30 minutes, rinsing the coupons in deionized water. Coupons were then placed in a sterile biosafety cabinet to dry. Once dry, the coupons were then stored in disinfected 5 gallon buckets.

Figure 5. Cement-lined coupon with spore suspension

3.3.1 Tap water conditioning

Coupons were exposed to an open channel of Cincinnati tap water by placing them into a 5.9 meter long 25.4 cm diameter PVC pipe cut in half length wise as shown in Fig. 6. The top of the pipe was cut in half to allow access to the coupons inside. Tap water entered the device at approximately 3.5 liters per minute at a pH which ranged from 8.26 to 8.87 with a median of 8.62. The average chlorine concentration of the incoming tap water was 0.94 with a standard deviation of 0.10.

Figure 6. Coupons conditioning in Cincinnati tap water (note that covers have been removed for photo and iron coupons were kept downstream from other coupon materials so the corrosion would not contaminate them)

Coupons were conditioned in Cincinnati tap water for approximately 13 months prior to experimentation. To determine the level of biofilm growth, coupons were brushed with a toothbrush to remove any biofilm using 40 mL of phosphate buffer as a diluent (Standard Methods Section 9216 B; (A.D. Eaton, 2005) The resulting suspension was diluted and spread-plated on R2A agar (Remel Inc., Lexana, KS catalog no. R01722.) When no colonies were detected on the biofilm samples from cement-lined and PVC coupons, a confirmation was done one these samples using a fluorescence microscope and the FilmTracer™ LIVE/DEAD® viability kit (Invitrogen Inc., Carlsbad, CA, Catalog no. L10316).

3.3.2 Bare coupons

New coupons were sprayed with a 5% sodium hypochlorite solution and then were placed into 5 liter buckets of autoclaved Cincinnati tap water 24 hours prior to use in experiments. This was done to hydrate the coupon surfaces in the same water that was used to make the spore suspensions. Water was autoclaved to keep biofilm from forming on the coupons during the 24 hour period.

3.3.3 Tank conditioned coupons

Twenty-five coupons of each material used in the spore retention experiments for the bare and agarose "biofilm" experiments were placed in a pH adjusted solution of 10% sodium hypochlorite for 5 hours and were brushed 3 times to remove any material remaining on the coupon surfaces from the previous experiments. Two coupons of each pipe material were sampled to determine whether there were any residual spores from previous experiments left on the surface by placing each in a 200 mL sample container filled with 100 mL phosphate buffer with Tween 80. The container which was suspended in an ultrasonic cleaning bath was cleaned for 20 minutes at 40 kHz. The entire volume of phosphate buffer was then run through a membrane filter and plated as described in the following section. Ductile iron coupons were autoclaved after disinfection in sodium hypochlorite.

The cleaned and disinfected coupons were then suspended in a 1136-liter recirculation tank with a residence time of approximately 14 hours as described in (B. H. Packard & Kupferle, 2010)The average chlorine concentration of the incoming tap water was 0.99

mg/L with a standard deviation of 0.11 during the time period. Sodium thiosulfate was incrementally added to the tank with a chemical feed pump to reduce the chlorine concentration to less than 0.01 mg/L of detectable free chlorine in the tank. In order to enhance biofilm growth within the tank, the dechlorinated tap water was augmented with sodium acetate, sodium nitrate, and sodium phosphate which were continually added to the recirculation tank using a syringe pump to achieve concentrations of 1000 ug/L, 100 ug/L, and 100 ug/L respectively, of carbon, nitrogen, and phosphorous in the tank.

3.3.4 Agarose beads and artificial biofilm preparation

Agarose "biofilm" was used to compare spore adhesion to different surfaces and to assess the potential of agarose "biofilm" to act as a biofilm surrogate for future studies involving contaminant adhesion. Superporous agarose beads were made by adapting the method described by Strathmann *et al*.(Strathmann, *et al.*, 2000). Superporous agarose beads were prepared by mixing 4 grams of agarose (Agarose SFR™ number j234-1006, Amresco Solon, OH) with 400 mL of distilled water. The mixture was heated to 95º C on a hotplate and was allowed to cool to 50º C. A pre-warmed (50º C) mixture consisting of 3 mL Tween® 80 and 300 mL of cyclohexane was added to the agarose solution and mixed in a 1 L beaker at 1000 RPM with a laboratory mixer. The beaker was left on a hot plate to maintain the temperature. After 5 min., a second solution of 12 mL Span® 85 (Catalog number 57135-250 mL, Sigma, St. Louis, MO) and 300 mL cyclohexane (preheated to 50º C) was added to the emulsion and mixed at 500 RPM for 10 min. The emulsion was allowed to cool to room temperature while mixing at 500 RPM. Beads were allowed to settle, and excess cyclohexane was decanted off. The beads were then

washed twice by mixing them in DI water, allowing them to settle and decanting the water from the container. DI water was added to the container to store the beads and the container was stored in a laboratory refrigerator at 4 ^{o}C.

Before agarose beads were applied to the coupons, 0.4 mL of a 70º C 2% solution of agarose (without beads) were pipetted onto the coupon surface and spread with a flat laboratory spatula so that the agarose covered the entire surface of the coupon. Immediately after spreading the agarose, agarose beads (1±0.12 g) were then placed on the coupon surface using a laboratory spoon (Fisher Scientific, Pittsburgh, PA, catalog no. 14-375-10). The coupon was then left to cool for 5 minutes to allow the agarose to solidify. The coupons were then rinsed with 50 mL of dechlorinated tap water in order to rinse off any beads that were not fixed to the surface as well as rehydrate the surface prior to storing the coupon. Rinsed coupons were placed in sealed containers and were not removed until they were used in the experiments.

3.3.5 Spore preparation, growth, storage, and enumeration

BG spores were prepared, grown, stored, and enumerated as described in (Packard & Kupferle, 2009). Prior to experimentation, a 10^3 CFU/ml spore suspension was made with DI water and 1 mL aliquots of the suspension were pipetted into multiple 1.5 mL sterile tubes which were kept frozen at -20º C until use. On a given experimental day, spore suspensions to be used were made from the frozen stock by adding one 1 mL of the stock to 100 mL of tap water dechlorinated with sodium thiosulfate. The resulting

suspension containing approximately 8.6 x 10^3 spores per mL. Coupons were leveled, inoculated with 2 mL of the suspension, and then left for 20 minutes.

4.3.6 Coupon Inoculation and Rinsing

Prior to inoculating the coupons, the sides and backs of the coupons were wiped dry with a sterile paper towel. Then the coupons were leveled, inoculated with 2 mL of the suspension, and left for 20 minutes. After the 20 minute exposure to the spore suspension, the residual suspension on the coupon was poured into a 250 mL sample container. The coupon was then placed in a buret stand as shown in Fig. 7 with the 250 mL sample container beneath it. The coupon was rinsed with 200 mL of dechlorinated tap water pumped at 3.13 mL/second onto the coupon using a peristaltic pump and 25 mL polycarbonate syringe modified as shown in Fig. 7. Spores that were not retained by the coupon surface were removed in the rinse. The epoxy ridge around the perimeter of the coupon kept the suspension from running off prior to rinsing and the bottom of the Petri dish was visually inspected for liquid to ensure that there was no spore suspension that might have leaked from the surface to the bottom of the coupon. Samples that leaked prior to rinsing were not included in the evaluated data. Corroded ductile iron coupons proved to be the hardest to level and inoculate due to irregular roughness of the tuberculation on the coupons. For this reason, fewer iron coupons were analyzed than PVC and cement-lined.

Figure 7. Coupon rinsing apparatus using buret stand

4.3.7 Spore Retention Determination

Since the spore suspensions were made from the same stock solution, similar concentrations of spores were present on each coupon (17,640 ±2,129). To minimize propagation of error in comparison of results, counts taken directly from the rinse suspensions described above were compared to examine spore retention by the different pipe materials.

4.3.8 Pipe Material Surface Characterization

Surface roughness was measured using a KLA Tencor P-10 surface profilometer for bare PVC, cement-lined, and iron (non-corroded) pipe coupons. The profilometer works best on flat, dry surfaces without a large vertical deflection, so measuring the surface roughness of the conditioned coupons using this tool was impractical. However, the conditioned coupons were assessed with a scanning electron microscope (ESEM) to visually characterize the surface of the conditioned coupons. For this analysis, coupons were cut into 2 cm pieces that were spiked with 0.2 mL of tap water containing 10^7 spores/mL. The spiked coupons were placed in a biosafety cabinet until dry. The dried spiked coupons were rinsed (as described in the rinsing section) with 50 mL of dechlorinated tap water to remove any weakly attached spores from the surface, and the rinsed coupons were placed in a sterile petri dish and allowed to dry overnight. Coupons were then visually observed using an environmental scanning electron microscope in environmental mode (Phillips XL30 ESEM, FEI Co. a subsidiary of Phillips, Hillsboro, OR.). Coupon surface topography as well as location of spores was observed and photographed.

4.3.9 *Experimental Design*

In the first phase, the three pipe materials were randomly divided across the three surface preparation techniques. Within each group of 84 coupons, 28 were assigned to the different surface preparations (bare, tap water conditioned, and agarose “biofilm”) and four were used as biofilm and procedural blank samples (Table 1).

To determine the differences in spore retention between augmented "natural" biofilm and agarose "biofilm" in the second phase, 6 coupons of each pipe material (each with either augmented "natural" or agarose "biofilm") were compared to determine the difference in spore retention with respect to spore concentration. Three concentrations of spores ($6x10^3$, $5.8x10^4$, and $1.3x10^6$ CFU/ mL) were applied to the coupons and plate counts were calculated as described previously. Statistical t-tests were then used to determine the extent to which the two biofilms differed.

3.3.6 Statistical Analysis and Calculations

The experiments were designed for a two-way ANOVA to compare plate counts with a 95% confidence interval. The ANOVAs were carried out using plate counts using SigmaPlot® Software (SYSTAT Software, Inc., San Jose, CA). Since samples were randomized, the Holm-Sidak multiple comparison procedure was used at the 0.05 significance level to compare the factors.

3.4 Results

3.4.1 Surface Analysis

Both the bare PVC and cement surfaces analyzed for surface roughness were smooth to the touch with the exception of the ductile iron coupon which was noticeably rougher. Dry surface measurements showed that the three materials had similar Ra values as shown in Table 6. However, the roughness height measurement (Rh) showed iron to be different than the other materials.

Table 6. Average roughness and height of PVC, cement-lined, and iron coupons.

	Roughness (Ra) *a*		Roughness (Rh) *b*		
	Ra (µm)	SD	Rh (µm)	SD	*n*
PVC	0.98	0.68	-2.57	5.93	3
Cement-lined	0.88	0.41	-5.67	7.59	3
Iron	0.89	0.49	8.44	14.27	3

a Ra, arithmetic average deviation of the absolute value of the roughness profile from the mean line or centerline
b. Rh, height of the irregularities with respect to a reference line

Surface topography of the conditioned materials as visualized by the ESEM images (Fig. 8) showed the cement surface to be rougher with what looked to be sand particles protruding from the surface. Cavities along with microcracks were also observed, increasing the available surface area where spores could potentially accumulate if they were to make contact with the surface. In contrast, the PVC surface was homogenous and smoother with fewer areas for spores to be retained. However, ESEM images of the conditioned iron surface, showed the corroded iron surface to have much higher porosity and roughness than PVC and cement, increasing the available surface area of the coupon and providing a host of potential habitats for microorganisms to cling to. This supposition was supported by heterotrophic plate count data.

HPC counts for conditioned PVC and cement-lined coupons showed no viable growth which was confirmed with the LIVE/DEAD® viability kit. HPC analysis of iron coupons showed 3.9 X 10^6 CFU/ cm^2 to be present on the surface.

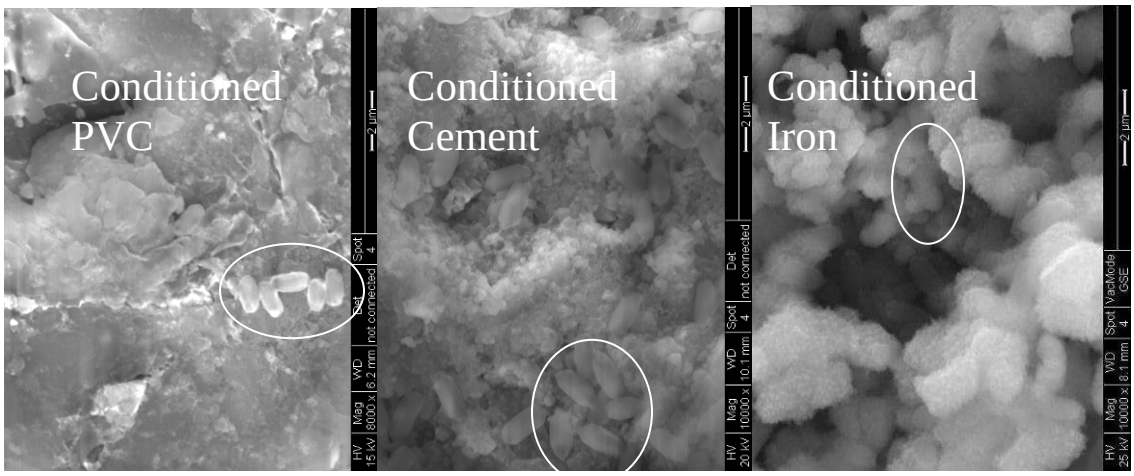


Figure 8. ESEM images of spores retained on conditioned PVC, cement, and ductile iron pipe coupons at 10000X magnification. Circles indicate examples of attached spores.

3.4.2 Plate Count Comparison for Spores Released from Bare, Conditioned and Agarose-Coated Coupons

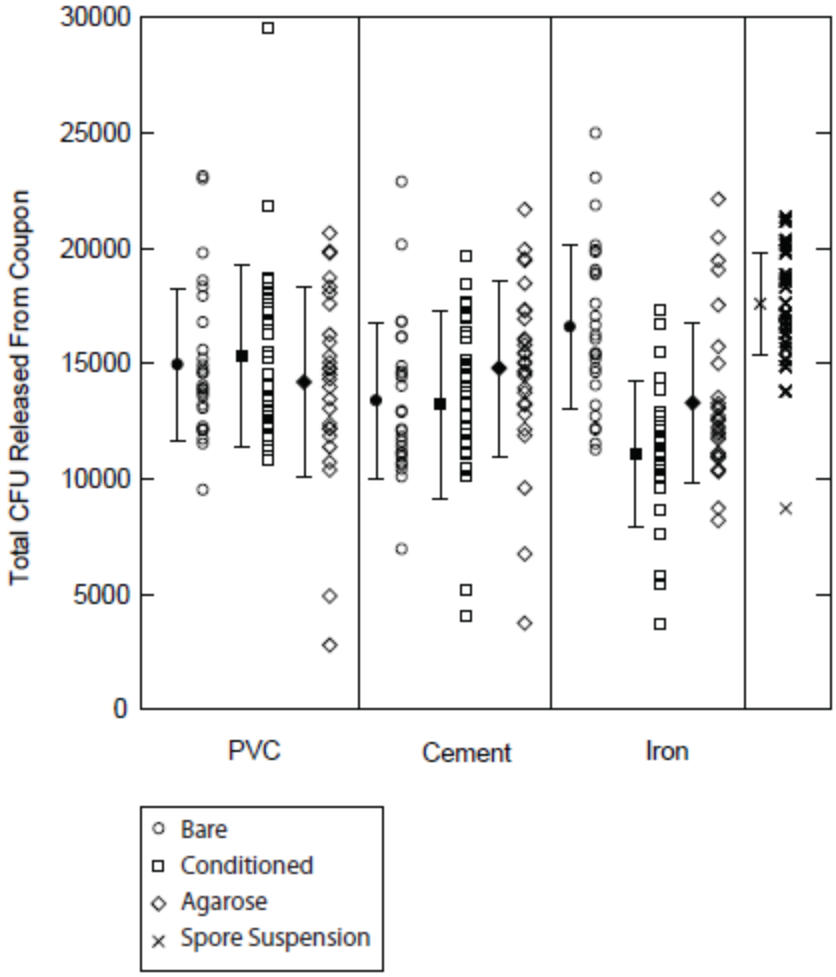
By conducting a mass balance for all data, overall spore retention for was 25.9 % with a standard deviation of 17.8%. Higher plate count numbers coincided with fewer spores being retained on the surfaces of the coupons. Two-way analysis of variance between pipe materials and surface treatments showed significant interactions between the surface preparation procedures (bare, conditioned, and agarose-coated) and pipe materials. Standard deviations between samples ranged from 15.7% for conditioned iron to 20.8% for PVC with agarose "biofilm". Table 7 shows significant differences between rinse plate counts for the different surfaces tested using the Holm-Sidak multiple comparison

procedure. Fig. 9 shows the difference between treatments where the Y axis is total number of spores released from coupon in the rinse procedure.

Table 7. Significant differences between rinse plate counts using the Holm-Sidak multiple comparison procedure.

Comparison (mean CFU recovered / mL)	P value
All bare combined (74.3) vs. all conditioned combined (66.0)	0.004
Bare iron (82.4) vs. bare cement (66.2)	0.004
Conditioned PVC (76.7) vs. conditioned iron (55.3)	<0.001
Conditioned cement (65.6) vs. conditioned iron (55.3)	0.039
Bare iron (82.4) vs. conditioned iron (55.3)	<0.001
Bare iron(82.4) vs. agarose-coated on iron(66.8)	0.004
Conditioned iron (55.3) vs. agarose-coated on iron (66.8)	0.021

Figure 9. Point plot of rinse plate counts (note that point on bars left of data indicates the column mean and bars represent one standard deviation)



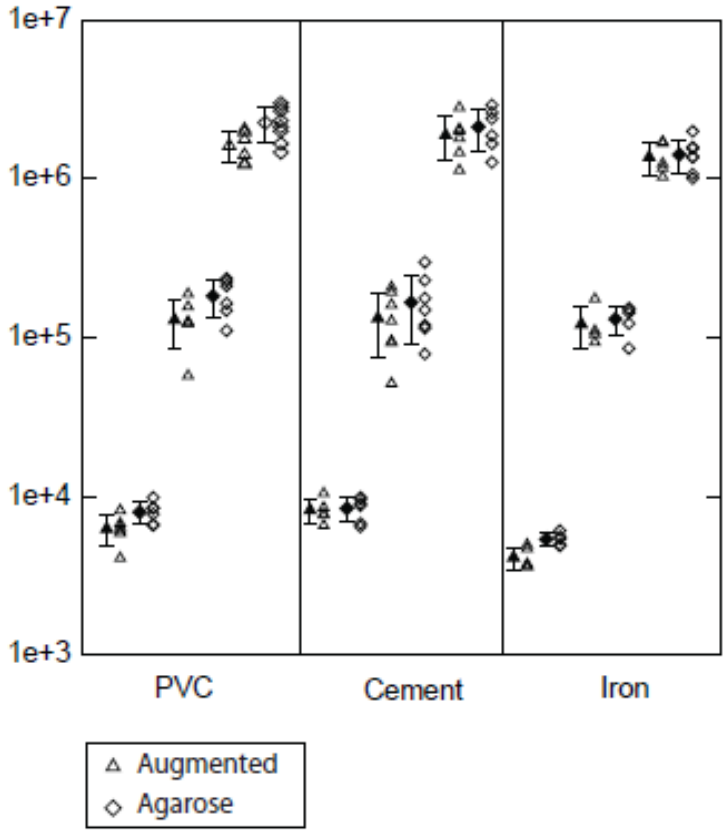
3.4.3 Agarose "Biofilm" Evaluation Experiments

Heterotrophic plate counts (HPC) for tank conditioned coupons conditioned in augmented dechlorinated tap water were found to have 1.3 X 10^6 CFU/cm^2 (PVC), 1.5 X 10^7 CFU/cm^2 (cement), and 3.8 X 10^7 CFU/cm^2 (iron). Comparisons of spore retention between agarose "biofilm" and augmented "natural" biofilm showed significant differences between the two for combined pipe materials at each concentration. Differences were also seen within the pipe materials at the different concentrations for PVC and iron as indicated in Table 8 and Fig. 10.

Table 8. Mean, standard deviation, and t-test P value of agarose "biofilm" vs. augmented "natural" biofilm

		No. of samples	Mean (sd)	No. of samples	Mean (sd)	Significant difference (p value)
6 X 10^3	PVC	6	6211 (1322)	6	8211(1272)	Yes (0.009)
	Cement-lined	5	8147(642)	6	9389(1629)	No
	Iron	5	4107(3.2)	5	5720(519)	Yes (0.48)
5.8 X 10^4	PVC	6	1.29E+05 (4.34E+04)	6	1.89E+05 (5.12E+04)	No
	Cement-lined	7	1.32E+05 (5.71E+04)	7	1.86E+05 (8.46E+04)	No
	Iron	4	3.69E+04 (1.21E+05)	5	1.34E+05 (2.85E+04)	No
1.3 X 10^6	PVC	8	1.60E+06 (3.48E+05)	6	2.34E+06 (5.53E+05)	Yes(0.008)
	Cement-lined	6	1.88E+06 (5.74E+05)	6	2.11E+06 (6.19E+05)	No
	Iron	5	1.37E+06 (3.10E+05)	7	1.52E+06 (3.58E+05)	No

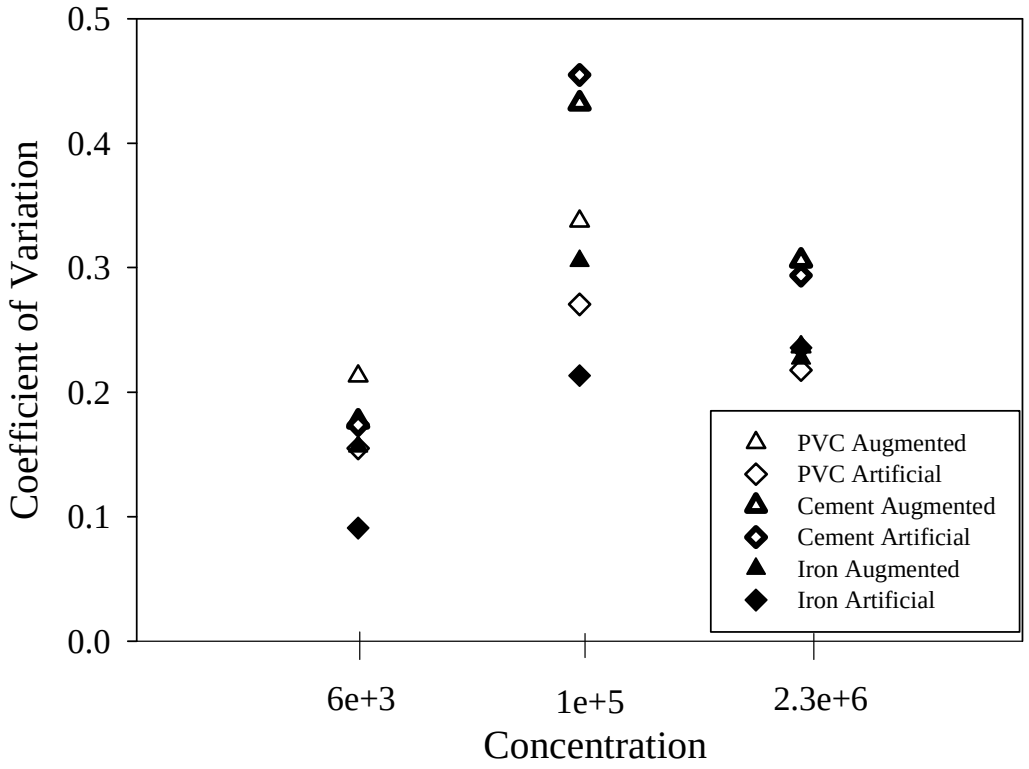
Figure 10. Agarose vs. augmented biofilm assessment at three different spore concentrations



Different concentration levels required different sample plating preparation to obtain CFUs in countable ranges, so there was concern that this could impact variability. At the 6 X 10^3 concentration 1 mL of each 200 mL rinse sample was membrane filtered. To enumerate the 5.8 X 10^4 and 1.3 X 10^6 concentrations, 0.1 mL of the rinse samples were spread plated with the 1.3 X 10^6 concentration diluted 10:1 prior to plating.

The coefficient of variation was calculated for each concentration. Fig. 11 shows variability was slightly higher for the samples at the medium spore concentration, but there is no apparent bias with respect to coupon type/surface treatment.

Figure 11.Coefficient of variation for agarose "biofilm" assessment rinse data



3.5 Discussion and Conclusions

In the first phase of the study, the concentration of spores spiked to the coupons was the same, allowing the same procedure for rinsing and spore enumeration to be used for all of the samples. Experiments showed conditioned iron to retain significantly more spores than conditioned PVC, bare cement, bare iron, and iron with agarose "biofilm" as shown in Table 7. These differences between the iron and the other materials were expected due to the observable layer of tuberculation and corrosion on the conditioned iron coupons.

The large amount of corrosion as compared to the cement-lined and PVC coupons likely provided more surface area for retaining the spores. Visual comparisons between the pipe materials validate this supposition as iron has visibly more surface roughness and, consequently, more area.

In addition to aiding in retention, iron coupons with agarose "biofilm" had higher numbers of adhered spores as compared the bare iron coupons. Rust, generated in the 24-hr hydration period, seeped into the agarose (discoloration was noted on the surface of the agarose), indicating that the rust was integrated into the matrix. In contrast to the iron coupons with biofilm, bare iron had lowest spore retention. This could be explained by the hydrophilic surface properties of the iron which would not have enhanced adhesion of the hydrophobic spores. It may also have been due to the thin layer of rust that formed during the 24-hr conditioning period. During rinsing, rust from the surface was visually observed to wash off, possibly bringing with it any attached spores. In contrast, the cement-lined coupons, which are also hydrophilic, did not have the layer of rust. This may explain the significantly higher spore retention on the bare cement-lined coupons as compared to the bare iron coupons.

Agarose "biofilm" evaluation experiments showed significant differences between the agarose-coated coupons and coupons with the augmented "natural" biofilm. At each spore concentration, comparisons between agarose and augmented biofilm for all pipe materials combined showed that augmented "natural" biofilm retained more spores (indicated by lower plate counts) than did the agarose "biofilm". Despite these

differences, spore retention between the two biofilms was within the same order of magnitude, which meant that the same serial dilution procedure could be used for both, making the two data sets more readily comparable (Benjamin H. Packard & Kupferle, 2009).

Comparisons between pipe materials for the agarose evaluation experiments further validated the ability of the iron coupons to retain more spores than the other materials. For two out of three of the spore concentrations, iron coupons (both agarose and augmented “natural”) tended to have higher spore retention than PVC and cement coupons. This was expected due to the iron corrosion and biofilm matrix. Moreover, iron with agarose had higher spore retention as seen by the differences between iron and cement/PVC coupons at the low concentration and iron and PVC at the high concentration. Therefore, adding another substance such as iron to the agarose matrix could possibly enhance spore retention. If agarose “biofilm” is used for future experiments, additional materials can be added to the agarose to more appropriately mimic the adhesive characteristics of living biofilms.

In contrast to the low concentration, the medium concentration did not show significant differences in spore retention for any of the pipe materials. One explanation for this might be the high variability for all the samples at the medium concentration as seen by higher coefficients of variation (Fig. 11).

In addition to iron retaining more spores, it also had the lowest variability. Standard deviations for iron coupons were consistently lower for both agarose and augmented biofilm as compared to the other pipe materials indicating that the presence of iron corrosion both absorbs more spores and also reliably release similar distributions of spores. This has been observed in previous experiments as well .

Iron coupons also showed significant differences at the lowest spore suspension between agarose and augmented biofilm. The observation that agarose retained more spores than augmented "natural" on iron was not expected given the difference in surface roughness and amount of corroded iron found on the augmented "natural" biofilm coupons as compared to the agarose "biofilm" iron coupons. This difference, however, was not seen at the medium and high spore concentrations.

In contrast to iron, cement coupons had the highest variability which was not expected due to the porosity of the surface and higher concentration of augmented "natural" biofilm present. Significant differences which were observed between for PVC coupons but not observed with cement-lined coupons may have to do with the increased biofilm growth on cement as compared to the PVC coupons ($1.3 \text{ X } 10^6$ CFU/cm^2 vs. $1.5 \text{ X } 10^7$ CFU/cm^2 respectively). There might not have been enough augmented biofilm on the PVC coupons to mask any effects from the pipe material. Since augmented "natural" biofilm on cement-lined coupons did not show a difference, it could be implied that agarose does not represent augmented "natural" biofilm at lower concentrations such as was measured on the PVC coupons, but will represent augmented "natural" biofilm at

higher concentrations such as the higher level of biofilm that was measured on the cement coupons.

Alternatively, if the material surface properties did heavily influence spore retention, results should have shown more spores adhering to both the agarose and augmented "natural" biofilm for cement-lined coupons to due to the increased biofilm, porosity, and roughness. Since this was not the case, it could be concluded that spore retention on biofilm covered surfaces is more dependent on the presence of biofilm, rather than the surface properties of the underlying material. The exception to this might be if corrosion from the underlying surfaces is integrated into the biofilm as was the case with iron. If this is the case, the chemical properties of the biofilm may change, which can impact spore retention.

For the spore plate count comparison for spores released from bare, conditioned and agarose-coated coupons, only in the case of iron did the conditioned coupons differ from the bare coupons. It was suspected that the 13 months of conditioning in Cincinnati tap water might impact spore retention due to the effects of water contact on the new cement lining (*Internal Corrosion of Water Distribution Systems*, 1996) Even if the cement lining formed calcium carbonate deposits as is the case for cement exposed to water, this was not shown to impact spore retention.

The reported data illustrate the capacity of corroded iron to retain spores more readily than other materials. Furthermore, the data highlight the importance of selecting a

possible replacement for naturally grown biofilm for contaminant retention experiments. Agarose has been shown to simulate natural biofilm as well as underwater surfaces reasonably well as seen by the comparisons between agarose and augmented “natural” biofilm on cement-lined coupons. Since one of the observations was the increased spore retention of iron integrated into agarose, further research should investigate additional materials that could be added to agarose to increase or decrease its potential to retain contaminants of concern.

CHAPTER 4: Conclusions and Recommendations

The reported research illustrates the ability of bacterial endospores to contaminate PVC, cement-lined, and ductile iron pipe materials. The following are major conclusions from these studies:

1. Both the physiochemical properties of the pipe material along with the presence or absence of biofilm on that material will impact spore retention.
2. The variability in the number of spores retained on the surface increased on PVC and decreased on ductile iron. Sampling recovery followed similar patterns.
3. Corroded iron presents the worst-case contamination scenario in that it significantly retained more spores than PVC and cement-lined pipe material.
4. To achieve accurate recovery of *Bacillus* spores, a surfactant such as Tween® 80 should be added to reagent water used in the assays.
5. The technique best suited for surface sampling wetted pipe material is dependent on the surface. Porous surfaces such as cement should be brushed; non-porous surfaces should be scraped.
6. The technique best suited for sampling corroded iron surfaces is dependent on the nature of the corrosion. If the corrosion is loose and easily removable,

scraping will be the best method. If the pipe is tuberculated the best technique may be to brush the outer layer of corrosion from the tuberculation.

7. Conditioning PVC and cement-lined pipe material in tap water for 13 months did not increase spore retention.
8. Spore retention on agarose artificial “biofilm” was enhanced by iron corrosion.
9. Agarose biofilm may mimic the properties of living biofilm with respect to spore retention, but spore retention on agarose is influenced by the underlying substrate properties.

The forces described in the Chapter 1 which influenced spore retention were transport of spore to surface (i.e. flow), physiochemical characteristics of the spore and the wetted surface (i.e. hydrophobicity and surface charge), and the water chemistry (i.e. pH and ionic strength of the water). It is still not evident which of the forces most influences spore retention. However, the results of the phase II plate count comparisons of spores released from bare, conditioned, and agarose-coated coupons (each surface having a different surface chemistry) showed no major differences between the PVC and cement-lined coupons. If the surface chemistry of the pipe material (i.e. hydrophobicity and charge) were important factors, significant differences should have been observed, especially with the high number of samples taken. Since the spore suspension was placed on the coupon surface where it sat stationary, the only mechanism for spores to make contact with the surface over time would been to gravity settling. Had there been mixing or flow within the suspension

as would be the case in a drinking water pipe, spores may have been transported to or sheared from the pipe surface more efficiently, and might have shown differences in retention between pipe materials. The difference between conditioned iron coupons and the other materials showed that the surface roughness, porosity, and surface area of the conditioned iron coupons enhanced retention. The chemical properties of the oxidized iron might have also enhanced spore retention. From these observations, surfaced roughness, or total surface available along with transport seem to be the more important factors influencing spore adhesion.

If a distribution system is contaminated with a persistent microbiological agent, there will be strategies for removing the contaminated bulk water and decontaminating the internal surfaces. One of the keys to being able to return the system to full use will be to be able to quickly assess the efficacy of the decontamination taken to accurately assess the level of contamination left in the system. Thus, surface sampling may be the only way to accurately gauge the levels of residual contamination after the system is decontaminated. With this in mind, the following are recommendations for future research areas which were identified during the process of planning and conducting the research in this thesis:

1. Analysis of conditioned cement-lined pipe to determine the “contamination potential” for various persistent contaminants in addition to spores.
2. Determination of the ability of cement-lined, PVC, and tuberculated iron pipe surfaces to be decontaminated from a range of contaminants. It may be the

case that certain contaminants are retained on the various pipe materials at unacceptable levels even after decontamination of the surface. Knowledge of problematic pipe material/contaminant combinations ahead of time is critical to making a quick determination as to whether to decontaminate or immediately replace contaminated pipe.

3. Due to the large amounts of iron corrosion that may be present in samples, methods selected to quantify contamination should be tested to determine how iron corrosion impacts recovery.
4. A standard protocol for surface sampling *in situ* in the distribution system should be developed by adapting the sampling techniques and methods developed in this thesis. An important aspect of the protocol will be to specify numbers of samples that provide the statistical power to accurately assess whether the system is clean.
5. Analysis of biofilm grown in low-nutrient, high shear conditions should be continued to determine the levels of the various constituents (protein, carbohydrate, DNA, etc.) making up the biofilm as well as the morphology of the biofilm. These parameters should be related to water quality, hydraulics, and pipe material in the system to assist in contamination risk assessment models.
6. Development of an artificial biofilm that is similar chemically and physically to living biofilms should be continued. Entanglement gels composed of multiple constituents, i.e. proteins and carbohydrates, in addition to crosslinked carbohydrate molecules like agarose gel should be considered.

CHAPTER 5: References

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Appendix A

Quality Assurance Project Plan (QAPP)

Spore Adhesion to Drinking Water Pipe Material

Submitted by:

Benjamin Packard
Water Infrastructure Protection Division
National Homeland Security Research Center
U.S. Environmental Protection Agency

Approvals:

Benjamin Packard, Principal Investigator/WIPD	Date
Kim R. Fox, Director/WIPD	Date
Eletha Brady-Roberts, Director of Quality Assurance/NHSRC	Date

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0.0 DISTRIBUTION LIST

Kim Fox, Division Director, Water Infrastructure Protection Division, NHSRC
Eletha Brady-Roberts, Director of Quality Assurance, NHSRC
Jeff Szabo, Environmental Engineer, Water Infrastructure Protection Division, NHSRC
Alan Lindquist, Microbiologist, Water Infrastructure Protection Division, NHSRC
Margaret Kupferle, Professor of Environmental Engineering, University of Cincinnati

1.0 PROJECT DESCRIPTION AND OBJECTIVES

1.1 Purpose of study

In the past decade, especially following 9/11 and the anthrax mailings, the possibility of persistent biological agents being used to shut down critical infrastructure has taken on increasing concern. Persistent biological agents require extensive and lengthy cleanups once they are distributed into the environment, and therefore, they have the potential to disrupt public health and the economic vitality of an area. *Bacillus* spores are recalcitrant and are able to stay viable in water for two years even in the presence of increased levels of chlorine (Watson and Keir, 1994) (Maginnis et al., 2001). Endospores present another problem: they persist for long periods of time following an event. In light of the multiple impacts intentional contamination would have on a community, it is important to assess how to accurately sample water infrastructure in order to determine the extent of contamination as well as the efficacy of the decontamination of the system. The objective of this project is to quantify the spores that attach to the internal surfaces of infrastructure, given different parameters and pipe materials.

1.2 Process Description

The U.S. Environmental Protection Agency's National Homeland Security Research Center (NHSRC) in Cincinnati will conduct a study to investigate *Bacillus* spore attachment to drinking water pipe coupons. Because of the many variables involved in conducting risk assessments and modeling contamination scenarios of drinking water infrastructure, data are needed regarding the contamination potential of *Bacillus* spores. This study will provide data by examining spore adhesion to internal pipe surfaces.

This study will evaluate the impact of water parameters, surface characteristics, and contaminant characteristics on spore adhesion. These factors are listed below, along with values that are typically found in a distribution system.

Table 1: Proposed study variables

Variable	Ionic strength			Contact Time		Concentration	
				Pulse injection vs. continuously fed		Varying	
Reason for study	Liquids with higher ionic strengths will cause particles to flocculate or adhere to a surface.			Contact time and concentration will vary depending on the threat scenario. It is assumed that higher concentrations and contact times will result in higher levels of spore adhesion.			
System averages	EPA MCL for Total Dissolved Solids = 500 mg/L ≈ 350 µS/cm (conductivity measurement)			Unknown			
Proposed variables	50 µS/cm	250 µS/cm	450 µS/cm	1 hr	24 hrs	10^2 CFU/mL	10^5 CFU/mL

	Surface Characteristics					
	Pipe material			Biofilm quantitation methods		
Variable	Unlined cast and ductile iron	Cement lined cast and ductile iron	PVC	Protein (µg/cm^2)	Carbohydrate (µg/cm^2)	HPC (CFU/cm^2)
Reason for study	Internal pipe surface material will exhibit varying characteristics with respect to surface roughness, surface charge, and level of biofilm growth.			Biofilm is ubiquitous in drinking water systems. Pathogens have been shown to adhere to biofilm.		
System averages	18%	50%	8%	1 to 4	1 to 5	10^3 cm^2
Proposed variables	Unlined ductile iron, concrete-lined ductile iron, and PVC			Biofilm will be established on new pipe coupons and will be preserved on pipe coupons taken from a distribution system.		

1.3 Project Objectives

The objective of this project is to investigate the following:

- Compare the sensitivity and percent recovery of pipe surface sampling methods
- Determine differences in *Bacillus* spore adhesion to real-world pipe coupons given differences in ionic strength, spore concentration, and spore contact time
- Investigate protein and carbohydrate quantitation methods for biofilm on pipe surfaces

2.0 PROJECT ORGANIZATION

2.1 Personnel

Table 2 Project participants and functions

Name	Phone	Email	Function
Kim Fox	(513) 569-7820	fox.kim@epa.gov	Division Director
Eletha Brady-Roberts	(515) 569-7662	roberts.eletha@epa.gov	QA Manager
Margaret Kupferle, PhD	(513) 556-3329	margaret.kupferle@uc.edu	U.C. Advisor for thesis
Jeff Szabo, PhD	(513) 556-2823	szabo.jeff@epa.gov	EPA Advisor
Alan Lindquist, PhD	(513) 569-7192	lindquist.alan@epa.gov	EPA Advisor
Ben Packard	(513) 569-7324	packard.benjamin@epa.gov	Technical Support/Principal Investigator

2.2 EPA Quality Assurance

Eletha Brady-Roberts, Director of Quality Assurance for NHSRC, will oversee project quality requirements for the EPA. As the Director of Quality Assurance, she serves in the Immediate Office of the Director of the NHSRC and is independent of project management.

2.3 Responsibilities of Project Participants

Ben Packard will be responsible for writing the QAPP and related Standard Operating Procedures (SOPs). He will also order supplies, maintain the laboratory, and ensure that the laboratory efforts are performed in a careful and controlled manner consistent with the QAPP. He will direct the preparation of media and reagents, perform the collection of data, and carry out analysis of that data. He will be in charge of modifying the study as required in order to obtain data that are conclusive and useful.

No specialized training or certification is required for any of the procedures involved in this project beyond the standard education and experience required for microbiology laboratory work.

Dr. Kupferle, Dr. Szabo, and Dr. Lindquist will act as advisors to Ben Packard throughout the project. They will review the QAPP and will check to see that data generated is consistent with the QAPP and QA Plan.

2.4 Facility

The experiments will take place at EPA's research facilities in Cincinnati: the Andrew W. Breidenbach Environmental Research Center (AWBERC) and the Testing and Evaluation Facility. The work will be conducted in two phases. The first phase will investigate pipe sampling procedures. This phase will focus on new pipe materials and the goal will be to test surface sampling methods to determine the precision and percent recovery of the method. The second phase will incorporate pipe recently collected from real-world distribution systems. The natural pipe surface will be preserved and will be exposed to a variety of spore suspensions.

3.0 EXPERIMENTAL APPROACH

3.1 General Approach and Test Conditions

This study will be split the experiments into two phases. Both phases will involve exposing pipe coupons with biofilm to spore suspensions. In Phase 1, the metrics of interest will be the determination of the total number of spores that adhere to the coupon and the percent recovery associated with sampling techniques. Phase 2 will determine the numbers of spores that adhere to the coupon surface, given differences in contact time, concentration, and ionic strength. The corrosion on the internal surface of the excavated pipe may differ from the newer pipe surfaces used in Phase 1. Therefore, a judgment will be made on the spot to determine the best method of sampling the excavated pipe section.

The experimental setup will consist of pipe coupons (3 cm by 10 cm) cut from new and used 6- to 10-inch water mains. One of the requirements for Phase 2 will be to cut coupons from recently excavated water mains in order to preserve the natural biofilm and aged pipe surface of the coupon. In Phase 2, pipe will be collected directly from the site, sampled, and transported to the machine shop where coupons will be cut out and then transported to the lab to be used for experimentation. Currently it is unknown whether biofilm on the coupon would be dislodged in the cutting process. Therefore, samples will be taken prior to and after cutting in an effort to determine the extent of disruption. Since biofilm is not uniform within pipes in a distribution system, biofilm samples taken right after the pipe is excavated may not be representative of biofilm within the pipe or on coupons cut from the pipe section. Thus, all coupons that are used in Phase 2 cannot be assumed to have similar amounts of biofilm with each other and the level of disruption in the cutting process cannot be definitively determined. The sampling technique to be used in Phase 2 will depend on the level of corrosion and surface properties of the excavated pipe and also from data from Phase 1. Depending on the porosity and the level of corrosion and biofilm, the surfaces will either be scraped or brushed to remove spores or biofilm. A 3 x 10 cm sample will be taken from the excavated pipe. Four samples from top, bottom, and sides will be taken immediately after the pipes are excavated. HPC counts from the four samples will be compared and the variability between samples will be analyzed. Once coupons are cut out of the pipe, they will be placed in groups of four with the internal pipe surface of each flush with the others. Trays will hold the coupons on wire supports so that the internal surfaces of the coupons are only making contact with the spore suspensions. Magnetic stirrers will be used to keep the spores from gravity settling in the containers. Magnetic stirrers will not be used to generate turbulence, sheer forces, or any type of flow within the tray. The purpose of using this setup is to isolate the variables that impact spore adhesion at the solid-liquid interface.

Statistical analysis for both phases will be conducted using an analysis of variance between the different sampling techniques in Phase 1 and the different spore exposure schemes in Phase 2.

3.1.1 Phase 1

The goal of Phase 1 will be to compare brushing and scraping to determine which is the most effective in removing spores from the coupon. The result will be a determination of which sampling method is the most reliable given a certain type of pipe material. The metric of interest will be percent recovery of spores from the surface of the coupon. Phase 1 will use coupons cut from cement-lined, PVC, and ductile iron water main. The procedure for Phase 1 is outlined in appendix B. Percent recovery will be calculated as indicated in section 3.4.1.

Because biofilm is ubiquitous in a drinking water distribution system, an effort will be made to condition the pipe coupons with a natural biofilm prior to conducting the experiments. This will be done by exposing coupons to dechlorinated Cincinnati tap water for approximately three months prior to the experiment. Coupons will be hung in a 300 gallon recirculation tank to condition the coupons and establish biofilm on the surface. Biofilm on the surfaces will be characterized by measuring heterotrophic plate counts, total protein, and total carbohydrate.

Sampling efficiency results from Phase 1 will provide information that will help to choose the best sampling technique to be used in Phase 2. Scale from the excavated water mains might differ considerably from the biofilm/scale that will be found on the new Phase 1 coupons. Culture methods will be used for spore and biofilm enumeration in both phases.

3.1.2 Phase 2

Phase 2 consists of exposing real-world pipe coupons to a variety of spore suspensions to determine the relative impact of each. Coupons for this phase will be cut from sections of pipe taken from the Cincinnati Water Works distribution system. The impact of increased ionic strength, spore concentration, and spore suspension contact time on spore adhesion will be measured. The pipe material used for these experiments will depend on what material is in need of repair in the distributions system and what is available. Coupons will be sampled using techniques shown to give the highest percent recovery and precision in Phase 1. In the event that pipe is not available from the water utility, new pipe coupons will be conditioned in the same manner that is to be done in Phase 1.

3.1.3 Pipe Coupons

New and used pipe sections will be cut using a high-pressure water jet cutter. This method of cutting uses a very small high-pressure jet of water and abrasive material that can cut through up to 6 inches of steel while keeping the surface and the metal cool as it is cut. Dimensions for the cut are programmed into a computer that controls the machine. For Phase 1, coupons will be cut with the water jet cutter, then washed and conditioned. They will then be placed in a 300 gallon recirculation tank prior to being used in the experiments. For Phase 2, coupons will be cut with the high pressure water jet method to preserve the biofilm and scale from the excavated pipe section. It is estimated that a

coupon that is 3x13 cm^2 will be large enough that the cut edge will not dislodge the majority of the biofilm and corrosion. The first coupon that is cut from pipe obtained from the distribution system will be inspected to determine the extent of disruption to the biofilm and scale within the pipe. Coupon size may be altered depending on the level of disruption of the scale/biofilm in the cutting process. Once Phase 2 coupons are cut, they will be placed in ice in a cooler and stored at 4–6ºC to be transported back to the lab to be used in the experiments. The Phase 2 coupons will be kept wet by spraying coupons with spray bottle containing dechlorinated tap water as soon as they start to dry out.

Pipe material selection for Phase 1 is based on the current statistics supplied by the American Water Works Association (AWWA, 2002). This will include concrete lined, PVC, and unlined ductile iron pipe sections. The 39 cm^2 of sampling surface on a 3x13 cm^2 coupon should provide enough surface area to get representative samples of biofilm and spores. Prior to use, new coupons will be conditioned using the National Sanitation Foundation procedure for conditioning drinking water contact materials prior to determining the toxicity of the surface (ANSI/NSF, 2000). This process is outlined in Appendix B.

3.1.4 Spore Exposure Setup

Pipe coupons will be exposed to tap water to establish a conditioning film on the coupons prior to the Phase 1 experiments then the coupons will be inoculated with spore suspensions and then sampled to obtain sampling efficiency data. Glass trays will be used to contain the spore suspensions for Phase 2. The trays will be placed on magnetic stirrers set on very low rates to ensure that suspensions do not settle over time. Temperature will be measured hourly to ensure that the stirrers do not heat up the suspensions. If it is found that the suspensions are increasing in temperature, reusable gel ice packs will be placed around the sides of the trays. The trays will then be monitored for temperature to ensure that the suspensions do not heat up due to the stirrer. Wire will support the coupons so that only the internal pipe will be making contact with the spore suspensions. Coupon surfaces will be submerged roughly 2 mm into the spore suspension.

3.2 Methods

Methods for spore and biofilm enumeration are listed in Appendix A for protein and carbohydrate analysis, and plating procedures for biofilm and spores are listed in Standard Methods 9215 and 9218 respectively. Media and reagent water preparation procedures are listed in Appendix C. The protein and carbohydrate biofilm enumeration methods will be assessed prior to being used to generate data in Phase 1 or 2. This assessment is described in Appendix A

3.2.1 Biofilm Growth

In Phase 1, a biofilm will be grown on the coupon surfaces by exposing the coupons to unchlorinated tap water in a 300 gallon recirculation tank for 2-3 months. The levels of HPCs on the coupons surfaces will be determined immediately prior to use. Phase 2

coupons will be cut from pipe excavated from the Cincinnati Water Works distribution system and the natural biofilm on the surfaces will be preserved. If excavated pipe is not available, new pipe coupons will conditioned the same way as they will be in Phase 1 but the coupons will be exposed to chlorinated tap water.

3.2.2 Biofilm Analysis Enumeration

Biofilm will be characterized using heterotrophic plate counts. Depending on the feasibility of the methods, protein and carbohydrate quantification assays may be used in addition to culture. An estimate of 2–3 $\mu g/cm^2$ for protein and 3–4 $\mu g/cm^2$ of carbohydrate are present on drinking water distribution system biofilms. These estimates will be at the lower limit of methodological resolution and, therefore, will not be the primary means of quantifying the biofilm but will add another dimension to the characterization of the biofilm by providing information on the ratio of protein which is prevalent in the cells to starch or carbohydrate, which is prevalent in the extracellular polymeric substances (Langmark et al., 2005).

The assay to be used to quantify the polysaccharide levels in the biofilm will be the Colorimetric Method for Determination of Sugars and Related Substances (Dubois et al., 1956). This method uses concentrated sulfuric acid along with a small amount of phenol to produce a color change in the sample that can be read at 485 nm on a spectrophotometer. Samples are enumerated using a standard curve that uses glucose as the standard. The ratio of acid to sample in the original Dubois study was about 70% acid to 20% sample. A modification to the assay by Kimberley and Taylor (1995) found that changing the acid/sample ratio to 80% and 20%, respectively, and adding a cooling step after the addition of the acid produced increased absorbance readings. Therefore, the Kimberley/Taylor modification of the method will be used to quantify carbohydrate.

Proteins found in biofilms are indicative of cell mass along with a portion of the material present in the extracellular matrix. Therefore, protein quantification assays may also be used to quantify the total amount of protein in the biofilm samples and may provide comparisons of protein to carbohydrate ratios in biofilms from different areas of a distribution system. The Coomassie Plus protein quantitation kit will be used to quantitate protein in the biofilm samples. Samples with high levels of pipe scale will yield correct results due to the results from the work described in Appendix A. All biofilm will be extracted from the pipe using sterile phosphate buffer using the sampling techniques described in Appendix B.

If there is enough biofilm sample left, a 10:1 dilution will be made and will be run through a TOC analyzer. The values will be compared to TOC measurements for the Cincinnati tap water to determine the TOC from the biofilm. TOC analysis will depend on the availability of biofilm samples and the TOC analyzer.

3.2.3 Bacterial Selection / Spore Development

Studies conducted to determine spore-forming *Bacillus* species that could be used as surrogates for *B. anthracis* have found that the mean *CT* (*C* is the concentration of chlorine in mg/liter, and *T* is the exposure time in minutes) values for *B. globigii* are higher than the corresponding mean CT values for the three other surrogates and the virulent strain of *B. anthracis*, making *B. globigii* a good surrogate for persistence and disinfection studies (Rice et al., 2005). *B. globigii* serves as a conservative surrogate for inactivation studies using chlorine. Thus, data generated from this project could be used in future studies involving disinfection. *B. globigii* spores will be grown as indicated in Appendix C.

3.2.4 Spore Enumeration / Viability / Accuracy Checking

B. globigii spores will be enumerated using spread plate procedures described in section 9215 of Standard Methods for the Examination of Water and Wastewater (Clesceri et al., 2005).. The media for spore enumeration will be tripticase soy agar plates as specified in Standard Method 9218 A. Aerobic Endospores When dilutions of samples are needed, sterile buffer (0.05 M KH_2PO_4), which is described in Appendix C, will be used as the diluent. Colony forming units (CFU) will be counted to approximate the numbers of viable spores sampled from the coupon. Percent recovery will be calculated as described in section 3.4.

3.2.5 Water Quality Monitoring

The following parameters will be measured in all dechlorinated tap water used for spore suspensions or rinse water before and after the experiments. Temperature will be monitored throughout the experiments to ensure that the temperature stays within ± 5º C of room temperature (~25º C).

- pH
- Conductivity
- Temperature

If time, resources, and equipment permit, alkalinity, nitrate, phosphorous, dissolved oxygen, and total organic carbon may be measured to characterize the water.

3.2.6 Water Parameter Manipulation

Water of differing ionic strengths will be required for Phase 2. To change the ionic strength of the suspension, equal amounts of KH_2PO_4, NaCl, KCl, and $BaCl_2$ will be dissolved in tap water. The conductivity of the suspension will be measured in order to quantify the ionic strength of the suspension. Conductivities of 50, 250, and 450 µS/cm will be established by incrementally adding a concentrated solution of the salts listed above (Berg and Sanjaghsaz, 1995; Lytle et al., 1999).

3.3 Experimental Design

Relative percent recoveries of brushing and scraping will be assessed in Phase 1. Phase 2 will use the same sampling procedures on the real world pipe samples as was done in Phase 1. Phase 2 will consist of a series of experiments to determine the impact of ionic strength, contact time, and spore concentration on the raw number of spores that adhere to the coupon. Analysis of variance will be used for both Phase 1 and 2 to determine overall significance of the spore recovery method (Phase 1) and spores adhering to the coupon (Phase 2) by observing the differences in percent recovery and spores recovered. Paired comparisons among individual recovery methods and spore recoveries will be performed to achieve an experimental type I error of 0.05.

3.3.1 Phase 1 Experiment

Phase 1 experiments will use 16 coupons of each pipe material. Sampling techniques and pipe materials to be used in Phase 1 are outlined in the following table:

Table 3: Phase 1 - Direct inoculation of spore suspension onto coupon to evaluate sampling techniques

	Procedural Blank (Coupon spiked buffer containing no spores)	Brushing	Scraping	TOTAL
PVC	1	8	8	17
Cement Lined	1	8	8	17
Iron	1	8	8	17
				51

3.3.2 Phase 2 Experiments

Phase 2 experiments will assess the adhesion of spores to excavated water main sections using the sampling technique (brushing or scraping) that was found to yield the highest percent recovery in Phase 1. Additionally, coupon inoculation tests identical to those conducted in Phase 1 will be conducted if there are enough coupons to conduct this test. As with Phase 1, the predicted number of coupons for each spore suspension treatment will be three and will depend on the amount of pipe that was collected from the distribution system, available time, and lab space. The goal will be to conduct three separate Phase 2 experiments, each on a different pipe section collected from the distribution system. Parameters to be tested in Phase 2 are outlined in the following table:

Table 4: Phase 2 - Coupon exposure experiments

24 hour spore exposure		Spore Concentration		TOTAL
		10^2/mL	10^5/ml	
Ionic strength	50 µS/cm	3	3	6
	250 µS/cm	3	3	6
	450 µS/cm	3	3	6
				18

1 hour spore exposure		Spore Concentration		TOTAL
		10^2/mL	10^5/ml	
Ionic strength	50 µS/cm	3	3	6
	250 µS/cm	3	3	6
	450 µS/cm	3	3	6
				18

	Procedural Blank (Coupon spiked buffer containing no spores)	Brushing	Scraping	TOTAL
Available section	3	3	3	9

Note that pipe material may vary depending on the availability of various pipe materials from local water utilities.

3.4 Statistics and Data Acquisition

3.4.1 Phase 1

The general test design for Phase 1 is to measure the concentration of spores that are removed from the coupon surface using one of the sampling techniques. A mass balance approach will be used to determine the percent recovery for each coupon by spiking the coupon with a known quantity of spores measured in colony forming units (CFU) per mL. After 16 minutes, the coupon will be rinsed with tap water which will be enumerated for spores. In order to determine the total number of spores left on the surface, the quantity of spores in the rinse will be subtracted from the initial quantity of spores applied to the coupon surface. Percent recovery will be calculated as follows:

C_i = the initial concentration of the spore suspension applied to the coupon
C_r = the concentration of rinse solution
C_a = the number of spores that adhere to the coupon surface
$\%C_a$ = the percentage of spores that adhere to the coupon surface
C_s = number of adhered spores recovered from the coupon surface
$\%C_s$ = the percentage recovery of adhered spores or the sampling efficiency

To determine $\%C_a$ and $\%C_s$, the following equations will be used:

$\%C_a = C_i - (C_r + C_{s\&b}) / C_s * 100$
$\%C_s = C_s/C_a \text{ X } 100$

Data will be saved in CFU/mL for C_i, C_r, and C_s. Following the experiments, C_a, $\%C_a$, and $\%C_s$ will be calculated using an Excel spread sheet. The experimental design will allow the following null (H_O) and alternate (H_A) hypothesis to be statistically tested:

H_O: $\%C_{s(i)} = \%C_{s(j)}$ for all i, j
H_A: $\%C_{s(i)} \neq \%C_{s(j)}$ for some $i \neq j$

The experimental design will enable testing of the null or alternate hypothesis by assuming that there will be no difference in percent recovery among the sampling techniques and pipe materials.

3.4.2 Phase 2 Data Analysis

For Phase 2, only one sampling method will be used and will be chosen based on percent recovery data generated in Phase 1. CFU will be measured to determine C_a, the number of spores that are sampled off of the surface, which will be the metric of interest. Additionally, percent recovery calculations in Phase 2 will be the same as Phase 1, where $\%C_s$ will be the metric of interest. Sets of coupons will be exposed to variations in ionic strength, contact time, and spore concentration, as shown in Table 4. These experiments will compare mean values obtained for the different conditions to determine whether there is a statistically significant difference between them. Only one sampling method will be used for each excavated pipe section. Depending on the surface of the coupons, the sampling method may not be based on the results from Phase 1 since the excavated pipe surfaces may not be comparable to the Phase 1 surfaces.

Statistical analysis will consist of evaluating whether there is a meaningful difference between spore recoveries for the different treatments with a 95% confidence interval.

The following null and alternative hypothesis will be statistically tested in Phase 2:

H_O: $C_{s(i)} = C_{s(j)}$ for all i, j
H_A: $C_{s(i)} \neq C_{s(j)}$ for some $i \neq j$

The experimental design will enable testing of the null or alternate hypothesis by assuming that there will be no difference in spore recovery among the various spore suspensions.

4.0 SAMPLING AND MEASUREMENT METHODS

4.1 Sampling Strategy

The purpose of sampling in Phase 1 will be to characterize different types of sampling methods and match them with pipe materials to determine the most consistent and effective sampling procedures. The procedures found to be the most reliable will be used in Phase 2.

The location of pipe samples excavated from the ground will depend on repair requirements by the utility. Therefore, as pipe sections become available, they will be sampled.

4.2 Sampling Spores From Coupons

Specific procedures for brushing and scraping as outlined in Appendix B, will be tested during Phase 1. If, during the course of the experiments, procedures in Appendix B need to be altered it will be revised accordingly. The following table summarizes the spore enumeration samples that will be taken for each coupon:

Table 5: Spore samples taken for each coupon in Phase 1 and Phase 2

Sample	Description	Reason for sample	Number of samples	
			Phase 1	Phase 2
Coupon rinse sample	Following inoculation, coupons will be rinsed with 100 mL of tap water to remove spores that have not adhered to the surface.	Quantify the number of spores that do not adhere to the coupon.	1	0
Coupon brush/scrape sample	Following rinsing, internal pipe surface of the coupon will be brushed/scraped	Quantify the number of spores that come off with the toothbrush.	1	1
Coupon sides and back sample	Following brushing/scraping, the sides and back of the coupon will be brushed.	Quantify the number of spores found on the sides and back of the coupon	1	1
Petri dish check	Determination of the number of spores that stay attached to the petri dishes during the rinse and removal stages.	Quantitation of number of organisms left on the dish after the rinse and removal steps	3 dishes: (rinse, removal, and sides/back checks)	2 dishes: (removal, and sides/back checks)
Brush and scraper check	Determination of the number of spores that stay attached to the brushes and scrapers	Quantitation of number of organisms left on the brush or scraper after removing spores from the coupon	2 brushes/scrapers: (removal and sides and back checks)	2 brushes/scrapers: (removal and sides and back checks)
TOTAL			8	6

All samples will be enumerated on TSA plates in triplicate. Thus, each coupon to be used in Phase 1 will require 24 plates and each coupon in Phase 2 will require 18 plates in addition to plates required for the QA samples.

4.3 Pipe Sample Excavation Procedure

Pipe samples obtained directly from a distribution system will be obtained from local utilities and will depend on the replacement requirements of the utilities. The procedure listed in Influence of Distribution System Infrastructure on Bacterial Regrowth (Clement et al., 2003) will be used for pipe excavation and sampling. This will consist of the following:

- Prior to cutting pipe, mark flow direction and top of pipe with spray paint.
- Take precautions to minimize disruption to the internal scale as the pipe is cut and removed from the ground by gently removing the pipe from the ground by hand, ensuring that the section stays level.
- Take two samples (one from either side) of the pipe surface as soon as possible following excavation by scraping within a 39 cm^2 template. Mark both locations with spray paint to make sure that the sampled surface is not used for a coupon.
- If there is access to the system, such as a faucet, collect a bulk water sample upstream from the pipe excavation area to determine HPC counts in the bulk using a 1 L container.
- Cap the pipe, using rubber end caps, and place in a cooler along with the biofilm samples.
- Transport pipe to a machine shop and have the coupons cut immediately. Use a spray bottle to keep the coupons surfaces moist.
- Transport cut coupons to the laboratory for Phase 2 experiments.

4.4 Frequency of Sampling

Coupons in Phase 1 will be sampled after 18 minutes of exposure to the suspension. Samples for Phase 2 will be taken following a 1 hour and 24 hour exposure to the suspensions. Extra coupons will be used for biofilm enumeration prior to spore exposure.

4.5 Sample Measurements

A list of the required documentation is provided in section 5.

4.6 Steady-state Conditions

All glassware and solutions, and media will be brought to the target temperatures prior to use in the experiments. All incubators and refrigerators will be brought to 35 ± 0.5º C and 4 ± 2º C 1 hour before being used. The same materials (brushes, vials, stock buffer solutions, media, reagents, and pipettes) will be used for each series of experiments to ensure that there is no variability.

Glassware, stir bars, graduated cylinders will be autoclaved. Equipment that cannot be sterilized in an autoclave will be submerged in a 1:1000 bleach solution (i.e., Clorox®) for 1 hour and rinsed three times with Milli-Q® treated water. Glassware and stir bars will be baked in a dry oven at 180ºC for three hours.

Procedures for using tap water are outlined in Appendix C. In addition, CFU will be assessed with respect to the variables given in Section 5, Table 7.

4.7 Site Preparation

All analyses will be performed on a bench top that has been disinfected with a 10% Clorox solution that has been adjusted to a pH of 6.0 to 6.5.

4.8 Cross-contamination

All sample containers will be labeled appropriately with name of sample, sample number, and date. All dilution blanks and spread plates will be labeled with sample number, dilution, date and time of day. Sterile disposable pipette tips will be used and discarded immediately after use. Spreading rods will be dipped in ethanol and burned in a flame after each plate is spread. Brushes will be discarded after being used. All solutions and glassware used in sampling biofilm or spores will be sterilized prior to use. Test tubes and volumetric glassware used to store and make standards for protein and carbohydrate quantitation assays will be acid washed between uses.

4.9 Representative Samples

Biofilm is variable within a distribution system and the amount of biofilm may depend on multiple factors as discussed in section 1.2. Levels may vary considerably within a 2-foot section of water main. Therefore, as pipe coupons are cut from sections of pipe, the level of disruption will be assessed to determine whether multiple locations on the pipe can be used to cut coupons. If possible, coupons be cut from top, sides, and bottom of the pipe if this is not possible due to disruption of pipe scale and biofilm from the initial cuts, coupons will only be cut from one of the sides of the pipe. Coupons will be marked to indicate where in the pipe they were taken from. For example, a top right side, a middle center, and a bottom left side coupon would be used to test spore adhesion or quantity of biofilm. After the coupons are sampled, the spore suspensions will be analyzed as soon as possible after the biofilm and spores are extracted from the coupon.

4.9.1 Sample Identification

Each sample tube, each dilution tube, and each spread plate will be pre-labeled with exposure time, pipe material, dilution, concentration, and date. The laboratory notebook and bench sheets will be filled out as the tests progress and data will be recorded as soon as they are read. Plate counts recorded onto bench sheets will be recorded in the laboratory notebook will be entered into an Excel file following the experiments.

5.0 TESTING AND MEASUREMENT PROTOCOLS

The following charts summarize the measurement protocols to be used for biofilm and spore enumeration as well as for water parameter sampling:

Table 6: Biofilm Sample Measurements

Parameter	Analytical method	Sampling procedure	Sample size/ container	Replicates	Preservation and storage	Maximum holding time
Heterotrophic Plate Count	*Standard Method*	Scraped from 38 cm^2 coupon into large Petri dish	NA	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Total Protein	NanoOrange® Protein quantitation kit	Scraped from 38 cm^2 coupon into large Petri dish	~0.1 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Total Protein	Coomassie Protein quantitation kit	Scraped from 38 cm^2 coupon into large Petri dish	~0.2 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Total Carbohydrate	Colorimetric Method for Determination of Sugars	Scraped from 38 cm^2 coupon into large Petri dish	~.5 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Total Organic Carbon	Tekman Dohrmann Phoenix 8000 UV persulfate TOC analyzer	Scraped from 38 cm^2 coupon into large Petri dish	~50 mL	3	Add 3 drops of H_2SO_4, and store at 4º C.	1 month

Table 7: Spore Enumeration Samples

Parameter	Analytical method	Sample procedure	Sample size/ container	Replicates	Preservation and storage	Maximum holding time
Spread plating	*Standard Method* 9215A/C	Scraped from 39 cm^2 into 50 ml vial	~.5 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours

Table 8: Water Quality Samples

Parameter	Analytical method	Reference	Sample size/ container	Preservation and storage	Maximum holding time
pH	pH-meter	*Standard Methods* 1992	In situ	NA	NA
Conductivity	Extech handheld conductivity and temperature meter		In situ	NA	NA
Temperature	Extech handheld conductivity and temperature meter	*Standard Methods* 2550	In situ	NA	NA
Total and free chlorine	Hach DR 2400 Spectrophotometer		50 mL beaker	NA	NA

During the course of the experiments, if one of the methods listed in the preceding charts proves to be unusable, another method may be used. If this is the case, new protocols will be chosen and the QAPP and will be updated accordingly.

6.0 QA OBJECTIVES

The following quality assurance objectives will be employed to ensure that the proper methodology is followed:

6.1 Quality Assurance/Quality Control

All analytical samples taken will include positive and negative controls to ensure that the samples that are analyzed are accurately defined. No fewer than three replicates will be taken to get representative samples. Samples that are outside of the limits of detection, or that do not meet the acceptance criteria required for the method, will be noted and checked for possible errors contributing to the result. Where possible, the tests will be repeated. Table 9 summarizes the QA samples to be taken for both phases of the project:

Table 9: QA/QC Criteria

Media	Measurement	QA/QC Check	Acceptance Criteria	Corrective Action	Frequency
Water Parameters	TOC	Blank	< detection limit	Remake standards	Every biofilm sample
		Check Standard	80-120% recovery	Retake samples	
	pH	Check against pH 7 buffer	± 0.2 pH units	Use different DO meter	Every sampling event
	Alkalinity	Negative control samples	6.9<pH<7.1	Recalibrate meter and recheck	When time and resources permig
	Conductivity	Check solutions of 200 mS and 2.00 mS	Reading ± 10% of expected value	Adjust meter	During Phase 2 experiments
	Free and total chlorine	Check tap water fresh from the system	Reading ± 10% of expected value (~1 mg/L)	Calibrate spectrophotometer/ replace reagents	Weekly when coupons are exposed to drinking water
	Temperature	Check thermometer against a calibrated thermometer	±0.5ºC	Note discrepancies	Weekly and during every sampling event
Coupon	Spore Enumeration	TSA Blank: Incubate plates	No observed growth	Remake plates	Every spore sampling event
		Spore Viability and quantitation: Positive Control plate spike (Known quantity of spores spiked onto TSA)	100-200 CFU/plate	Check spore suspension and dilution procedures	Every spore sampling event
		Buffer check: Plate buffer solution on TSA to determine sterility	No observed growth	Remake buffer/autoclave all glassware	Every spore sampling event
		Tap water check: Plate tap water used for growing biofilm, and rinsing/spiking coupons	No observed *B. globigii* growth	Sample tap water from tank a second time and autoclave all glassware	Every spore sampling event
		Matrix spike: Scrape biofilm from PVC, Iron, and cement coupons. Spike each with 1 mL of spore suspension to determine impact of corrosion on spore enumeration.	100-200 CFU/plate	Compare to counts without corrosion and note differences	Two replicates for each phase.
	Biofilm HPC	Buffer blank: Plate buffer solution on R2A plates	No observed growth	Remake buffer	Every biofilm sampling event
		R2A Blank: Incubate plates	No observed growth	Remake plates	Every biofilm sampling event
	Biofilm Protein - Coommassie protein assay	Blank	< Detection limit	Remake buffer and repeat	Every biofilm protein sampling event
		BSA Checkstandard (10 ug/mL)	80-120% Recovery	Check reagents and make new standard curve	Every biofilm protein sampling event
	Biofilm Carbohydrate - Phenol Carbohydrate assay	Blank	< Detection limit	Remake buffer and repeat	Every biofilm carbohydrate sampling event
		Glucose Checkstandard (10 ug/mL)	80-120% Recovery	Check reagents and make new standard curve	Every biofilm carbohydrate sampling event

6.2 Equipment

SOPs for the various assays will be kept in the laboratory along with the lab notebook. The laboratory notebook will provide a log of procedures for each experiment. Where possible, the appropriate SOP will be referred to unless deviated from in the experiment.

Equipment such as the pH meters will be used in accordance with the manufacturer's manuals for those pieces of equipment. Larger equipment (refrigerators, autoclaves) will be operated using the standard SOPs outlined in the facility SOP manual. Laboratory equipment and instrumentation will be checked for accuracy as directed in Standard Methods 9020 B. The following chart summarizes data requirements for equipment:

Table 10: Data Requirements

Data Quality Acceptable If…	**Corrective Action If Unacceptable**
All equipment, sensor, and meter calibrations are current.	Out-of-date calibrations will be corrected by recalibration or replacement of item and the analysis redone.
Reagents used are not past expiration date. Reagent grade chemicals are used. Microbiological grade agar used. Traceable standards. Negative controls are used to test for sterility.	Expired reagents will be replaced and the analysis redone. Reject chemicals/agar that is not proper grade.
Refrigerators, autoclaves, and incubators are operating at the required temperature.	If temperatures were not maintained within the required limits of the test, the equipment will be fixed or calibrated and the analysis will be redone.

The PI will ensure that equipment requiring calibration and monitoring is maintained in accordance with the manufacturer's guidelines and EPA requirements. The following chart summarizes calibration, use, and maintenance for the equipment to be used:

Table 11: Calibration, Use, and Maintenance of Equipment

Equipment	QA/QC	Frequency	Acceptable Criteria	Corrective Measures
Centrifuges	EPA centrifuges are under a Preventative Maintenance Agreement (PMA) and undergo an annual PM.	Quarterly or as described	Described in instrument manual or SOP	Described in instrument manual or SOP: All non-conformances are corrected before the instrument is put back to use.
Autoclaves	Autoclaves use tape with each batch that contains the date, time, length of cycle, and contents of the cycle. Autoclaves are under a PMA and undergo a quarterly PM.			
NanoPure water purification system	Check conductivity			
Temperature controlled devices	All water baths, refrigerators, freezers, etc. will be checked prior to and after use.			
Balances	Balances are calibrated monthly and annually serviced through the PMA.			
pH and dissolved oxygen meters	pH meters are calibrated daily in buffer.			
Micropipettes	Micropipettes are calibrated annually.	Annually or as described		
Tracking and assessment of media, reagents and supplies	All medium is purchased from a nationally recognized supplier. Negative and positive controls are included in each batch. Lot, date of receipt, and expiration dates are all recorded in the laboratory logbook for reagents and media.	As needed or when new chemicals are received	Ensure critical information is recorded.	Document if information is available; qualify any results that are suspect based on uncertainties with expiration dates.

6.3 Standards

Protein and carbohydrate assays will require that standard curves be made prior to the tests. Therefore standards will be prepared and tested the day prior to the sample analysis to ensure that reliable standards are available when samples are being analyzed.

6.4 Data Documentation

All documentation of testing and results shall be kept in a project-specific file. The laboratory notebook or laboratory bench sheets bench sheets will contain the following for each experiment:

- Date of test run
- Data from water parameter analysis (pH, temperature, total and free chlorine, etc.)
- Results from plates
- Results for negative control plates

Data will be entered into a Microsoft Excel Spreadsheet. In addition to laboratory bench sheets, procedures, protocols, and observations will be recorded directly in a laboratory notebook, which will be present during all experiments.

6.5 Data Review

The Quality Manager will review test records to identify and resolve any inconsistencies. The person performing the review will initial the data copy and date it. All the hard copies will be kept in a binder and all electronic data will saved on CDs.

6.6 Data Reporting / Reduction and Validation

- Free and total chlorine, TOC values will be reported in mg/L. This value is read directly from the spectrophotometer or TOC analyzer.

- Microbial enumeration will be reported as colony forming units per milliliter (CFU/mL) for spread plates. The value reported will be the average value of the CFU/mL from triplicate plates.

- pH is read directly from the pH meter and is defined at the negative log10 of the hydrogen ion concentration.

- Conductivity will be read directly from the conductivity meter and will be reported in microSiemens/cm (µS/cm)

- Temperature is read in degrees Celsius (ºC) directly from a thermometer.

- Protein and carbohydrate concentrations of biofilm are reported in micrograms per cm^2.

6.7 Deliverables

Reports will be prepared summarizing the results of the study. If the results from this study are compiled for publication, the report will proceed through NHSRC management review.

6.8 Data Validation

Data and calculations will be double checked to ensure that data were correctly recorded and transferred. All EPA data records management will be filed. Data generated from microplate readers and other instruments will be saved directly into Excel files. Data collected in the lab will be recorded on laboratory bench sheets and transferred to Excel files as soon as possible.

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Appendix A

BIOFILM ASSESSMENT STUDY

Prepared by: _____Date:_____

Approved by: _____Date:_____

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
NATIONAL HOMELAND SECURITY RESEARCH CENTER

0.0 Scope and Application

The purpose of this appendix is to give direction on conducting a preliminary and ranging study to assess procedures for growing, characterizing, and quantifying biofilm. Biofilm annular reactors (BARs) located in 783 Engineering Research Center (ERC) at the University of Cincinnati College of Civil and Environmental Engineering will be used to grow the biofilm. The biofilm will then be used to conduct tests to characterize the interferences that biofilm, pipe scale, and other laboratory reagents have on a test. Two types of biofilm will be grown: one with dechlorinated Cincinnati tap water and the other with the addition of phosphorus, nitrogen, and carbon sources to enhance the biofilm growth rate. Once the two varieties of biofilm are grown, they will be assessed for total protein, total carbohydrate, and phospholipid content, as well as HPC counts to quantify viable cells. These assessments will be conducted with the addition of various corrosion materials found in drinking water distribution systems in order to determine the impact that these substances have on biofilm assessment and quantification. In addition, Tween 80, a surfactant used in spore extraction assays, will be spiked into the sample to determine its impact on biofilm characterization and enumeration.

1.0 Health and Safety Warnings

- Observe all safety procedures discussed in the UC Chemical Hygiene Plans.
- Observe all safety procedures discussed in the T&E Standard Operating Procedures.
- Disinfect laboratory equipment and benches daily.
- Report all accidents to the UC safety manager.
- Follow all safety precautions and warnings on test kits containing reagents.
- Analyze and manipulate hazardous chemicals in a fume hood. This will include the use of phenol, which is toxic and can be absorbed through skin inhaled.
- Manipulate all corrosive and toxic chemicals under the chemical fume hood, and dispose of them in the proper manner.

MSDS sheets detailing the hazards of media and reagents are available in the laboratory.

1.1 Interferences

Chlorine from tap water used as reagent water may interfere with some of the chemicals used in some of the experiments. Sodium thiosulfate will be added to tap water that is fed into the reactors to deplete the chlorine residual.

2.0 Equipment and Supplies

Standard microbiology laboratory equipment, including a chemical fume hood, an autoclave, incubators, hot plates, stirrers, and spectrophotometers, will be required for this experiment. Specific equipment for each assay is listed under each method.

3.0 Experimental Approach

The approach for this study is to assess the accuracy and precision of two protein quantitation assays, one carbohydrate quantitation assay, the HPC assay, and three spore enumeration assays to determine the levels of interference from various materials that would be found in samples taken from water infrastructure. Biofilm grown under two different conditions will be compared using the protein, carbohydrate, and HPC methods.

4.0 Biofilm Estimates

In a paper investigating various methods used to measure and grow biofilm, Langmark et al. (2005), measured total protein, carbohydrate, and HPC counts per cm^2. Coupons were used to grow biofilm in annular reactors as well as modified robbins devices, which consisted of coupons inserted into drinking water pipes that could be taken out to sample the coupon. From that work, the following are estimates of how much protein carbohydrate, and heterotrophic bacteria there were in the biofilm samples:

Protein/Carbohydrate/HPC estimates (ug/cm^2)(CFU/cm2)	Protein (μ/cm^2)	Carb (μ/cm^2)	HPC (CFU/cm^2)
Langmark et al., 2005	1 – 4	1 – 5	10^3/cm^2
Estimate	2	3	1500

In light of the study conducted by Langmark, the following are conservative estimates for total protein, carbohydrate, and HPC counts that might be obtained from tests on steady state biofilm sampled from an annular reactor or other devices:

Protein/Carbohydrate/HPC estimates (38 cm^2 / 14 ml PBDW)	Protein (μ/mL)	Carb (μ/mL)	HPC (CFU/mL)
Estimated concentrations	5.4	8.1	4071.4

4.1 Protein assays available

There are a number of protein quantitation assays available. Many of them trace back to a paper by Lowry et al., (1951) describing a method for assessing total protein using Folin phenol reagent. Since then, companies such as Invitrogen have developed kits to quantify proteins in solution. The following is a chart from the Invitrogen Web site that summarizes assays available for quantifying proteins:

(From Invitrogen Web site: http://www.invitrogen.com/content.cfm?pageid=11111)

Assay	Abs/Em (nm) *	Sensitivity and Range	Speed	Through-put	Compatible with
Quant-iT	485/590	250 ng – 5 ug	5 min	High	• Reducing agents • Amines
EZQ	450/620	20 ng – 5 ug	1 hr	Medium	• Reducing agents • Detergents • Ampholytes • Chaotropes • Amines
NanoOrange®	485/590	20 ng – 2 µg	30 min	Medium	• Reducing agents • Amines
CBQCA	450/550	10 ng – 150 µg	1 hr	High	• Reducing agents • Amines
Fluoresc-amine	390/475	60 ng – 2.5 µg	1 hr	High	• Detergents
OPA	340/455	40 ng – 5 µg	1 hr	High	• Detergents
Bradford	595/---	20 µg – 300 µg	10 min	High	• Reducing agents • Detergents • Chaotropes • Amines
BCA	562/---	0.4 µg – 8 µg	2.5 hr	Medium	• Detergents • Chaotropes • Amines
Lowry	750/---	200 ng – 300 µg	40 min	Low	
UV absorption	205/280	2.8 µg – 420 µg			

4.1.1 For the purpose of this study, the NanoOrange® protein quantitation kig made by Invitrogen and the Coomassie Plus® protein quantitation kit made by Pierce will be assessed for precision, given the addition of substances that may interfere. The NanoOrange® assay (Molecular Probes, 2007) was chosen because of its use in previous studies involving biofilm protein assessment (Langmark et al., 2005). Additionally, the NanoOrange® protein quantitation kit is more sensitive than the Coomassie Plus® kit (10 ng vs. 1 ug of protein/ml) but may be more sensitive to interferences from substances such as detergents, which may make it less sensitive to Tween (Pierce, 2004).

4.1.2 The NanoOrange® kit allows detection of proteins in solution in concentrations between 10 ng/mL and 10 ug/mL. According to the paper written by Langmark, between 0 and 4 ug/cm^2 of biofilm was present on coupons taken from annular reactors. Therefore, a conservative estimate of total protein may be 1 μ/cm^2. Samples extracted from two 19-cm^2 slides in 14 ml of buffer will contain 2.71 ug of protein/ml, which is within the ranges of both protein assays.

4.2 Carbohydrate assay

4.2.1 The Colorimetric Method for Determination of Sugars and Related Substances (Dubois et al., 1956) will be used to determine total carbohydrate in the biofilm samples. This assay involves adding .05 ml phenol to 2 mL of sugar solution: 5 mL of concentrated sulfuric acid solution is then added rapidly to the colorimetric tube. After 10 minutes the tubes are shaken and heated, and then the color is read using a colorimeter. The carbohydrate content can be quantified by referencing a standard curve previously constructed for the particular sugar under examination. Underwood, Paterson, and Parkes (1995) conducted the Dubois carbohydrate test on intertidal sediments and found that 100 mM Na_2 EDTA mixed into 100-mg sediment samples and incubated for 20 minutes at 20ºC gave the best percentage recovery for the carbohydrate. Addition of EDTA may be assessed as a preliminary step to the carbohydrate analysis.

4.2.2 According to Dubois, the required amount of carbohydrate needed for the test is between 5 and 35 ug of sugar for every 1 ml sample. Therefore, at an estimated 8.14 ug of carbohydrate per ml, the biofilm that will potentially be collected is within the limits of detection for the Dubois assay. If lower levels of carbohydrate are obtained from the samples, methods for concentrating may be looked at.

4.3 Heterotrophic plate count (HPC)

4.3.1 HPC will be performed as described in section 9215A (9215C for spread plates) in Standard Methods (Clesceri et al., 2005). If 10^3 CFU/mL is expected, 10^{-3} dilution will be conducted.

4.4 Impact of corrosion materials on protein and carbohydrate assays

4.4.1 Materials that can commonly be found in water distribution systems will be assessed for their relative impact on protein,

carbohydrate, HPC, and PLP assays. Results as well as their spore enumeration assays. The following interfering compounds will be directly added to samples of biofilm for a qualitative assessment of the impact of each substance.

4.4.2 Impact from iron oxide: Iron oxides will be present in samples from corroded iron pipes. If iron surfaces are exposed to tap water, they will quickly oxidize. When sampling a pipe surface such as ductile iron or cast iron that is new or has been recently cleaned, there will inherently be oxidized iron in the sample. This may impact microbial detection and enumeration assays as well as the biofilm assessment assays described above. Therefore, a XX M solution of ferric chloride will be spiked into the biofilm and spore samples to determine its impacts.

4.4.3 Impact from iron tuberculation: Tuberculation from two different cast iron water mains will be collected and ground up using a mortar and pestle. Tuberculation of iron pipes contains other metals such as calcium and sulfur in addition to insoluble iron solids formed over time in the system through primary, secondary, and tertiary reactions. There are no standards for pipe scale, so a standard composition cannot be determined. Scale from cast iron pipe taken from two different distribution systems will be used to determine whether there are significant interferences from the real-world tuberculation. Portions of scale that may be found in a sample if manually brushing or scraping the pipe surface will be used. The resulting solution will then be added to samples containing biofilm and spores.

4.4.4 Impact from cement: Cementous material, especially new cement, leaches carbonates and hydroxide ions when exposed to water, especially soft water. If cement is scraped off, when manually sampling a pipe coupon, corrosion and cementous material may be present in the sample. This can potentially raise the pH, which will impact the biofilm assessment and spore enumeration assays. To determine the potential effects, concrete from concrete-lined ductile iron pipe will be crushed and spiked into the biofilm samples.

4.4.5 Impact from Tween 80: will be assessed by spiking each sample vial with .01% Tween 80. Tween could potentially be added to aid in extracting hydrophobic or negatively charged microbes from surfaces. This may interfere with protein and carbohydrate assessments of biofilm.

4.5 Impact of corrosion materials on spore enumeration methods given the time and resources, mass spectrometry, culture methods, and PCR will be assessed with and without the addition of the corrosion materials listed above and biofilm. These experiments will involve spiking biofilm samples taken from the BARs with the four interfering materials listed above. In addition, spores will be spiked into the samples and assays will be run to determine the number of spores present in the samples.

4.6 Differences in biofilm grown under two types of conditions will be assessed. One of the reactors will be run using dechlorinated Cincinnati tap water for six weeks to grow a steady-state biofilm, which will be used as the control. The other reactor will be spiked with 250 ug of acetate during the last week of growth to determine the impact of an increase in assimilable organic carbon available to the biofilm. The purpose of this part of the study is to evaluate the differences in protein, carbohydrate, fatty acid, and HPC concentrations in the two different biofilms. The goal will be to determine whether biofilm grown with Cincinnati tap water changes with respect to protein, carbohydrate, or cell numbers (PLP/HPC) when a short-term increase of assimilability carbon is available. Therefore, three days of 250 μg/L of acetate will be fed into the reactors prior to extracting the biofilm.

5.0 Study Design

This study will use a replicated factorial design to determine the impacts on biofilm enumeration and characterization. The following variables will be assessed in the first part of the study (see Table 1):

- Two types of biofilm
- Four biofilm assessment assays
- Four interference tests (iron oxide, iron tuberculation, cement, and Tween 80)

The second part of the study will use a similar design to assess the impacts of corrosion materials on spore enumeration assays. The following variables will be assessed in the second part of the study (see Table 2):

- Two types of biofilm (enhanced and natural)
- Four interference tests (iron, iron tuberculation, cement, and Tween 80)
- Three spore enumeration assays (culture, PCR, and Mass Spectrometry)

Table 1: Biofilm assessment experiments:

		No Biofilm	Biofilm/No Contaminant		Ferric Chloride		Iron Tuberculation		Cement		Calcium Hydroxide	
			E *	N**	E	N	E	N	E	N	E	N
Biofilm Assessment Method	**Protein quantitation**											
	NanoOrange® kit	3***	3	3	3	3	3	3	3	3	3	3
	Coomassie Plus® kit	3	3	3	3	3	3	3	3	3	3	3
	Carbohydrate quantitation											
	Dubois sulfuric acid and phenol colorimetric test	3	3	3	3	3	3	3	3	3	3	3
	Phospholipid											
	phospholipid fatty acid test	3	3	3	3	3	3	3	3	3	3	3
	Culture											
	Hetrotrophic plate count	3	3	3	3	3	3	3	3	3	3	3
* Biofilm grown with the addition of acetate, called enhanced growth (E)												
**Biofilm grown under natural growth conditions with no nutrients added (N)												

6.0 Sampling

6.1 Biofilm sampling procedure

6.1.1 Remove coupons from both reactors after six weeks.

6.1.2 Extract biofilm from coupons using a sterile cell scraper. Into 14 mL of PBDW.

6.1.3 Take four coupons from each reactor. Ensure that coupons are taken symmetrically, rather than four in a row. Remove biofilm from the four coupons so that there five 28 mL samples per reactor each containing four coupons worth of biofilm. Each of the samples will be treated as described in Section 4.4 (control, Tween, iron, cement, iron chloride).

The following tables illustrate the projected amounts of biofilm and how the biofilm will be allocated.

Table 2: Total biofilm amounts

Biofilm Amounts	Number of Coupons	Extraction Buffer/Coupon (mL)	Total Volume
Reactor A	20	7	140
Reactor B	20	7	140

Table 3: Biofilm allocations per reactor

Biofilm Amounts	Number of Coupons	Extraction Buffer/Coupon (mL)	Total Volume
Reactor A	20	7	140
Reactor B	20	7	140

Table 4: Biofilm assay requirements

Sample Allocations (per reactor)	Number of Samples	Sample Size (mL)	Volume Required (total)
NanoOrange® Assay	18	0.064	1.152
Coomassie Plus® Assay	18	0.75	13.5
Dubois Carbohydrate Assay	18	0.45	8.1
Phospholipid Fatty Acid Assay	18	4	72
Heterotrophic Plate Count Assay	18	1.5	27
			121.752

The following water quality parameters will be monitored in both reactors.

Table 5: Water parameter sampling (to be conducted daily throughout the biofilm growth period)

Parameter	Analytical Method and Name	Reference	Sample Container/ Quantity of Sample	Preservation and Storage	Maximum Holding Time
TOC	APHA 5310 TOC analyzer Shimadzu TOC-V csh	APHA (1992)	Glass vial with Teflon-lined lid 10mL	Acidification and storage at 4°C	10 days
pH	APHA 4500H+ pH-meter Accumet AP61	APHA (1992)	In situ	NA	NA
Alkalinity	HACH alkalinity test kit	HACH catalog 2063700	Graduated glass cylinder then 250mL glass Erlenmeyer/ 100mL sample	NA	NA
Nitrate	APHA 4500-NO_3^- (spectrophotometer at 220/275 nm)	APHA (1992)	250mL glass Erlenmeyer/quartz cell	NA	NA
Phosphorus	HACH phosphorus test kit low range	HACH catalog HCT121	16-mm sample glass vial	NA	NA
Dissolved oxygen	APHA 4500-O, Fisher brand* Traceable* Portable Dissolved Oxygen Meter	APHA (1992)	In situ	NA	NA
Temperature	APHA 2550, Fisher brand* Traceable* Portable Dissolved Oxygen Meter	APHA (1992)	In situ	NA	NA

7.0 Procedures

7.1 Reagent-grade water will conform to specifications in Standard Methods

7.2 Phosphate buffered dilution water (PBDW)

7.2.1 Assemble the following:

Potassium phosphate (KH_2PO_4) 0.34 g
Reagent-grade water ~1.0 L

7.2.2 Combine potassium phosphate and 500 mL of reagent-grade water and stir to dissolve in a 2-L bottle.
7.2.3 Adjust pH to 7.2 0.5 with 1 N NaOH.
7.2.4 Bring volume to 1 L with reagent-grade water. Stir to combine.
7.2.5 Autoclave for 20 minutes at 121ºC.
7.2.6 Store at 4ºC

7.3 Dechlorinated tap water (to be used for spore suspensions and in BARs to grow biofilm)

7.3.1 Collect tap water in large sterilized container. Ensure that tap is flushed until the temperature of the flowing water does not vary by more than 1ºC over a period of 1 minute.
7.3.2 Allow container to sit until chlorine residual has dissipated.
7.3.3 Characterize water parameters prior to use. Use same tap water source for each group of experiments.

7.4 Tween extraction buffer to be used to extract biofilm from coupons to determine interference from Tween:

7.4.1 Assemble the following:

PBDW	~1.0 L
Tween 80	10.0 mL (0.01%)

7.4.2 Fill 2-L bottle with 500 mL of PBDW.
7.4.3 Add 10 mL Tween 80.
7.4.4 Bring volume to 1 L with reagent-grade water. Stir to combine.

7.5 Biofilm growth water (for the addition of 250 ug C eq./L)

7.5.1 Assemble the following:

Sodium acetate (carbon source) CH3COONa •3H20
Dechlorinated tap water

7.5.2 Prepare a stock solution by adding 0.142 g of acetate to 250 mL of reagent water. After mixing the solution, prepare ten 50-ml conical tubes with 25 mL of the acetate solution. One will be added each day to the 10 L of tap water.

7.6 Heterotrophic plate counts (HPC) will be performed as described in section 9215A (9215C for spread plates) in Standard Methods.

7.7 Colorimetric Method for Determination of Sugars and Related Substances

7.7.1 Assemble the following:

Reagent-grade sulfuric acid with specific gravity of 1.84
Reagent-grade phenol (80% by weight, prepared by adding 20 mL of distilled water to 80 ml of phenol)
Fast-delivery 5-ml pipettes
Colorimetric tubes
0.02, 0.05, and 0.1 ml micropipettes and tips

7.7.2 Pipet .5 mL of sugar solution containing 1 and ~60 ug/mL of sugar into a colorimetric tube and 27 uL.

7.7.3 Transfer 2 mL of concentrated sulfuric acid rapidly on the sample surface to obtain good mixing then shake tubes to make sure the acid and sample are mixed. Transfer 27 uL 80% Phenol to the samples and then place them in a 25º to 30º water bath for 10 to 20 minutes. The color will be stable and the samples can be read for several hours.

7.7.4 The amount of carbohydrate may then be determined by reference to a standard curve. Absorbance, a is measured using the equation: As = absorbance, where As is a dimensionless ratio equal to log10 Tsolvent/Tsolution, T is the percent transmittance, b is the length of light path, expressed in cm and c is the concentration, in micrograms of sugar per milliliter of final volume.

7.8 NanoOrange® Protein Quantitation Kit

7.8.1 NanoOrange® kit reagents

NanoOrange® protein quantitation reagent (Component A)
NanoOrange® protein quantitation diluents (Component B)
BSA standard (Component C)
Reagent water

7.8.2 Assemble the following:

Mercury thermometer
Dry bath incubator
Microplate block
Black 96-well flat bottom microplates
Digital thermometer with dual "K" thermisters
1.5 mL flat-top microcentrifuge tubes
Standard disposable fluorescence cuvette

7.8.3 The NanoOrange® kit can measure proteins in solution at concentrations between 10 ng/mL and 10 ug/mL. Follow NanoOrange kit instructions to make the NanoOrange diluent. Transfer 30 uL of biofilm sample into the NanoOrange® working solution. Follow procedures on the kit for adding the NanoOrange reagent.

7.8.4 Sample volume requirements are ~0.1 ml of sample if using analyzing samples using fluorescence cuvettes (depending on

level of biofilm in the sample) sample requirements for microplates will be substantially less.

7.8.5 Read fluorescence using fluorescence cuvettes or microplates allowing excitation at 485 nm and capturing the emission at about 590 nm.

7.9 Coomassie Plus® Kit

7.9.1 Coomassie Reagents

Assay reagent
Albumin standard ampules

7.9.2 The Coomassie Plus® protein kit has a range of 1–25 mg/mL. Protein samples are mixed with assay reagent, incubated briefly, and the absorbance is measured at 595 nm.

7.10 Phospholipid Fatty Acid Assay

Phospholipid fatty acid analysis is a good indication of viable cell numbers. This procedure involves placing the biofilm sample in methanol, chloroform, and phosphate buffer solution, which is then analyzed using the EPA Fatty Acid Methyl Ester (FAME) Analysis, SOP#: 05.SOP-M.002.01. 4 mL of the 27 mL biofilm samples will be required to perform this assay. Furthermore, it will validate the information on cell counts gained through the HPC assays.

8.0 Waste management

Dispose of all samples, solvents, reagents, and laboratory wastes in accordance with the Laboratory's Waste Management Guidelines.

Appendix B

SPORE SAMPLING PROCEDURES

Prepared by: _____Date:_____

Approved by: _____Date:_____

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
NATIONAL HOMELAND SECURITY RESEARCH CENTER

0.0 Scope and Application

Techniques for sampling microbes from surfaces vary widely, and therefore, there is no universal or standardized way to sample microbes. Problems with extraction occur especially when the surface is porous. Care must be taken not to inactivate or rupture the cells of the organisms during the sample extraction process, which can happen if a sonicator is used. This procedure describes the plan to evaluate extraction methods to determine which is the most effective. There are several ways in which microbes can be extracted from a surface. It has been shown that procedures for collection of *Bacillus* spores using swabs, wipes, and vacuum socks on nonporous surfaces typically tend to underestimate the actual number of spores on the surface (Rose et al., 2004). This is most likely to be true in the case of sampling from a porous pipe surface such as concrete or corroded iron. This evaluation will determine the relative sampling efficiencies and methods of spore removal in order to determine which method will work the best for the follow on sample analysis. There will be two steps that will determine the sampling efficiency.

Given that the limits of detection for culture-based methods of cell enumeration, it is important that a method for spore extraction be precise. This preliminary study will assess the efficiencies of scraping, brushing, sonicating, and combinations of these to determine what methods work the best.

1.0 Summary of Method

This Appendix will explain procedures for physically removing spores from a pipe coupon cut from a water main. The procedures to be assessed are the following:

- Scraping the corrosion off the coupon using a cell scraper.
- Brushing the coupon with a brush

Percent recovery, accuracy, and the precision of these techniques will be statistically analyzed.

2.0 Health and Safety Warnings

The analyst must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

3.0 Cautions

3.1 Reagent standards must be prepared fresh on the day of the analysis.

3.2 Determination must be made within 48 hours of collection and sample stored at 4º C.

3.3 TSA plates must be read no more than 24 hours after the addition of spore suspensions.

3.4 Heat shocking of spores should last no longer than 10 min over 80º C

4.0 Interferences

4.1 Due to the surface properties of the pipe, periodically, extraction solutions will be heterogeneous and have clumps of corrosion. For biofilm samples that containing solids, samples will be homogenized using a tissue homogenizer prior to analysis.

5.0 Preparing Coupons and Spore Suspensions

5.1 Coupons cut from new sections of pipe will undergo the following conditioning process prior to adding coupons to the tank:

5.1.1 Scrub coupons in tap water, using a test tube brush to remove any miscellaneous debris.

5.1.2 Using a spray bottle, spray the coupons and racks used to hold coupons with 200 mg/L sodium hypochlorite solution, coating all surfaces of the coupon.

5.1.3 Let the coupons and racks stand for at least 30 minutes, and then rinse with tap water.

5.1.4 Place coupons in racks, again rinse with tap water, and then rinse with deionized water.

5.1.5 Immediately expose coupons to trays containing continuously circulating tap water in order to establish a conditioning biofilm.

6.0 Preparing for Coupon Spiking and Sampling

6.1 Ensure that the following items are turned on and are calibrated and the lab has been disinfected:

- Autoclave or steam sterilizer capable of achieving 121ºC (15 l)
- Hot water bath set to ~95º C
- Incubator set to 35ºC
- Disinfect all bench tops, and biosafety cabinets with .01% bleach solution

6.2 Assemble the following on a sterile bench

- Paper towels
- 100 mL sterile sample bottles
- Rinse bottles filled with 50 mL of a PBDW and .01% tween solution.
- Toothbrushes/cell scrapers
- Pipette, adjustable – 1–200 uL, 100–1000 uL with sterile pipette tips
- 10 mL sterile disposable pipettes
- 50 mL sterile disposable pipettes
- Vortex mixer
- 10^6 spore suspension – Spore suspension to inoculate coupon
- Certified timer
- Peristaltic pump. Ensure that pump is calibrated to ~ 100 mL /min
- Sterile 50 mL

Set coupon racks up and distribute equipment in its order of use

6.3 Assemble the following items are standing by on the bench prior to collecting coupon samples:

- Paper towels
- 10% bleach solution
- Sterile cell spreaders
- 150 mm TSA agar 100 mm petri dishes
- Blanks filled with 9 mL of PBDW.
- Pipette, adjustable – 1–200 uL, 100–1000 uL with sterile pipette tips
- Vortex mixer
- Sterile 500 mL beaker or flask
- 5 sterile 50 mL test tubes filled with PBDW and .01% tween

Make sure that plates and dilution blanks are labeled and staged in the order that they will be used. Prior to sampling and spiking coupons, make sure that the water bath is on and set to 95º C, the incubator is set to 35ºC and the autoclave is on. Sterilize all surfaces that are to be used and assemble equipment and consumable items described above on the bench or biosafety cabinet. Set up coupon racks, sample bottles, to be used for spore spiking, rinsing, and removal in the biosafety cabinet. Place all equipment described above in biosafety cabinet, sterile bench tops, or the autoclave tray. Take a ~ 500 mL sample of tap water for the tank. This will be used as rinse water and spore suspension to be used for spiking the coupons.

7.0 Procedure

7.1 Spore suspension

7.1.1 Collect 1 L of dechlorinated tap water from the tank in a sterile beaker. Take the sample back to the lab and fill a 100 mL sample bottle with 50 mL of the dechlorinated tap water. Spike 10µL of concentrated spore suspension into the sample bottle. This is the spore suspension to be used to spike coupons. Label the bottle accordingly.

7.2 Coupon Collection

7.2.1 Using a sterile autoclave tray, take a petri dish to the tank and carefully remove a coupon from the rack and place it in the petri dish while under water to ensure that the coupon is not exposed to the air. Place the coupon with the internal pipe surface facing up on the dish, place dishes in a sterile autoclave tray and take the tray back to the lab. Ensure that there is enough water in the dish to keep the coupon submerged.

7.3 Coupon Inoculation

7.3.1 Remove the coupon from the petri dish. Make sure that the majority of the water on the surface of the coupon is drained from the coupon surface by holding the coupon vertically over the petri dish for 30 seconds.

7.3.2 Once the surface has drained, place coupon on a new petri dish with. Using a small level, place paper towels under the sides of the dish so that the coupon is level in all directions. This step ensures that the spiking suspension stays on the coupon surface and does not run off.

7.3.3 Spike the surface with .5 mL of the suspension in a 2 inch area in the middle of the coupon. Carefully, spread the suspension to all areas of the coupon using a sterile pipette tip. Once the entire surface of the coupon is covered, set timer for 9 minutes and activate a countdown timer.

7.3.4 Prepare rinse water and the peristaltic pump for the rinse phase of the experiment.

7.3.5 After 9 minutes is up, spread the suspension over the coupon again with a sterile pipette tip.

7.4 Coupon Rinsing

7.4.1 After the coupon has been inoculated, pick up the coupon so that it is perpendicular with the petri dish. Pick up the coupon quickly so that the suspension on the top of the coupon goes onto the plate and does not go onto the sides of the coupon. Using tap water from a peristaltic pump rinse the coupon for ~ 1 min by holding the pump tube 1/4 inch from the top of the coupon while allowing

the water to run down the coupon into the same petri dish that the coupon was spiked in. Take care not to get water onto the sides of the coupon. Ensure that the coupon surface is evenly rinsed by moving the tube slowly back and forth ¼ inch about 1 second each way. Place the petri dish out of the way. Rinse the bottom of the coupon to rinse any residual spores that may have stuck to the bottom during rinsing.

7.5 Spore Extraction

7.5.1 Brushing

- Using a rinse bottle containing 50 mL of phosphate buffer containing .01% tween 80, brush the coupon using downward and diagonal strokes ensuring that all of the material on the coupon goes into the dish. Rinse the coupon surface thoroughly between brushing, collecting the rinse in the petri dish. Complete this process 4 times. Rinse the sides and back of the coupon with the sterile PBS, ensuring that all surfaces of the coupon have been rinsed. Use the remaining PBDW in the rinse bottle to rinse the brush thoroughly. Save the remaining residual PBDW in the rinse bottle for rinsing the plate when transferring the suspension to the sample bottle.

- Transfer the PBDW to a sterile 100 mL sample container. Pour the remaining PBDW that is in the rinse bottle into the petri dish to rinse the dish and pour the rinse into the sample container.

7.5.2 Scraping

- Using a sterile disposable cell scraper scrape the top layer of corrosion and biofilm into a sterile 15 cm petri dish. Ensure that all of the material that is scraped off the coupon surface is deposited into the petri dish. Scrape the coupon from both directions horizontally so that the biofilm/spores are scraped to the center of the coupon, then rinse the coupon the same as was done with the brush. Go through this process 4 times in the same manner that was done for brushing. Save the remaining residual PBDW in the rinse bottle for rinsing the plate when transferring the suspension to the sample bottle.

- Transfer the PBDW to a sterile 100 mL sample container. Pour the remaining PBDW that is in the rinse

bottle into the petri dish to rinse the dish and pour the rinse into the sample container.

7.5.3 Sides and back check

- Hold coupon vertically over a sterile petri dish. Using 50 mL of buffer in a rinse bottle, rinse the sides and back of the coupon and then gently brush the sides, bottom, top and back. Collect this into the sterile petri dish and transfer to a labeled sample bottle. Do not brush or rinse the internal surface of the pipe coupon.
- Make sure countertops are cleaned and gloves are replaced during the transition between coupons.

7.5.4 Brush and scraper spore check

- Place brush or scraper into a 50 mL test tube containing 10 mL of PBDW with .01 % tween.
- Vortex the tube containing the brush or scraper for 2 min using 10 second intervals.
- Ensure that any residual buffer is off of the brush by pressing the bristles against the side of the tube. Remove the brush/scraper and discard them.

7.5.5 Petri dish spore check

- After the spore suspension has been transferred to the sample bottle, swab the surface of the Petri dish with a moistened macrofoam swab.
- Swab the plate in one direction with one side of the swab turn the plat 90 degrees and swab the plate using the other side of the swab.
- Place the swab into a 50 mL sterile test tube containing 10 mL of PBDW containing .01% Tween.
- Vortex the tube containing the swab for 2 min using 10 second intervals.
- Ensure that any residual buffer is off of the swab by pressing it against the side of the tube. Remove the brush/scraper and discard them.

7.6 Spore Enumeration

7.6.1 Ensure that the plates are at room temperature and are properly labeled. The following is the plate labeling scheme:

- Pipe material: (Iron – I; Concrete – C; PVC – P)
- Action: (Scrape – S; Brush – B) (Rinse – R) (Sides/back – S/B)
- Dilution: (0; -1; -2; etc.)
- Prior to pipeting suspensions onto plates, double check that all material has been legibly labeled.

7.6.2 Following heat treatment, vortex the tubes and sample bottles solutions for 30 seconds. Make serial dilutions as required. Make sure that you stays organized so that samples are plated on the properly labeled petri dishes.

8.0 Quality Control and Quality Assurance

QA/QC samples and procedures discussed in section 6.0 will be followed.

9.0 Data Analysis

Data analysis procedures discussed in section 3 of the QAPP will be used.

10.0 Water Parameter Monitoring

Water parameters outlined in section 5.0 will be monitored for all tap water used in the study.

Appendix C

PREPARATION OF REAGENT WATER AND MEDIA

Prepared by: _____Date:_____

Approved by: _____Date:_____

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
NATIONAL HOMELAND SECURITY RESEARCH CENTER

0.0 Scope and Application

The procedures in this appendix specify how reagent water and reagents will be prepared. Reagent-grade chemicals shall be used in all tests. Included in this appendix are instructions on microbiological media preparation and instructions on how water with various ionic strength and pH values will be made. Lastly, in order to expedite biofilm growth, acetate will be added to tap water that is in contact with the coupons.

1.0 Health and Safety Warnings

- Observe all safety procedures discussed in the QAPP HASP in addition to any site-specific safety considerations.
- Disinfect laboratory equipment and benches daily.
- Report all accidents to the ORD safety manager.

MSDS sheets detailing the hazards of media and reagents are available in the laboratory

2.0 Reagent-grade Water

Reagent-grade water will conform to specifications in Standard Methods for the Examination of Water and Wastewater (21st ed.).

2.1 Phosphate buffered dilution water (PBDW)

2.1.1 Stock potassium phosphate solution

Potassium phosphate (KH_2PO_4)	0.34 g
Reagent-grade water	~1.0 L

2.1.2 Procedure:

- Combine potassium phosphate and 500 ml of deionized water and stir to dissolve in a 2 L bottle.
- Adjust pH to 7.2±0.5 with 19 N NaOH
- Bring volume to 1L with deionized water. Stir to combine.
- Autoclave for 20 minutes at 121º C.
- Store at 4ºC.

2.1.3 Stock magnesium chloride solution

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6 H_2O$)	81.1 g

Deionized water
1L

- Combine and stir until dissolved in a 2L bottle.
- Autoclave for 20 minutes at 121ºC.
- Store at 4ºC.

2.1.4 Working PBDW solution

Stock potassium phosphate solution
1.25 ml
Stock magnesium chloride solution
5.0ml
Deionized water
1L

2.1.5 Procedure

- Combine ingredients and stir to mix
- Autoclave at 121º C for 30 min
- Store at room temperature.

2.2 Dechlorinated Tap Water (to be used for spore suspensions and rinse water)

2.2.1 Collect tap water in large sterilized container. Ensure that tap is flushed until the temperature of the flowing water does not vary by more than 1ºC over a period of 1 minute.

2.2.2 Expose container for 2 hours to an ultraviolet lamp such as one found in a biological safety cabinet.

2.2.3 Characterize water parameters listed in the sampling section of the QAPP. Use the same tap water source for each group of experiments.

2.3 Extraction Buffer With Tween

2.3.1 Composition

PBDW	~1.0 L
Tween 80	0.1 mL (0.01%)

2.3.2 Fill 2-L bottle with 500 mL of PBDW.
2.3.3 Add pipet 100 ul of Tween 80.
2.3.4 Bring volume to 1 L with reagent-grade water. Stir to combine.

2.4 Water With Acetate (preparation of 1 liters)

2.4.1 Composition

Tap water	~1.0 L
Acetate	1.4 g

2.4.2 Add 1.4 g of acetate to 1 L tap water.
2.4.3 Mix thoroughly and place 1 mL of the solution into a second container holding ~ 999 ml of tap water.
2.4.4 The resulting solution will have 250 ug of carbon/mL.

2.5 TSA Media

2.5.1 Composition (preparation of 1 liter)

Nutrient Broth	8.0 g
$MnSO_4$	40.0 mg
$CaCl_2$	100.0 mg
Reagent-grade water	1.0 L

2.5.2 Combine ingredients in a 1-L flask and stir until dissolved.
2.5.3 Aliquot 100 ml into each of ten 500-ml flasks.
2.5.4 Autoclave flasks with media at 121ºC for 15 minutes.

2.6 R2A Media

2.6.1

Nutrient Broth	8.0 g
$MnSO_4$	40.0 mg
$CaCl_2$	100.0 mg
Reagent-grade water	1.0 L

2.6.2 Combine ingredients in a 1-L flask and stir until dissolved.
2.6.3 Aliquot 100 ml into each of ten 500-ml flasks.
2.6.4 Autoclave flasks with media at 121ºC for 15 minutes.

3.0 Spore Preparation Procedure

3.1 Slants of *Bacillus* on Heart Infusion (BHIA) agar are stored in the refrigerator. To prepare new spores, a fresh transfer of *B. anthracis* is streaked into a slant of BHIA and incubated overnight at 25ºC. This fresh culture is used to inoculate the spore media.

3.2 Growth of Spores

3.2.1 Inoculate 500 mL Erlenmeyer flasks containing 100 mL Generic Spore Media (see formulation below) with a culture of vegetable cells of *Bacillus.*

3.2.2 Incubate with continuous, gentle shaking at 35º for at least five days.

3.2.3 Check the solution for the presence of spores with a wet mount slide preparation using phase contrast microscopy.

3.2.4 When the slide preparation reveals an adequate spore suspension, proceed with the purification.

3.3 Purification of Spores

3.3.1 Ascetically transfer the contents of each flask into sterile 25-mL centrifuge tubes. Balance the tubes and centrifuge at approximately 5900 fcf for 20 minutes, using a fixed-angle rotor.

3.3.2 Pour off the supernatant into a discard beaker. Add 30 mL of cold, sterile deionizer water to each tube. Vortex each tube until the pellet is completely resuspended in the water. Centrifuge again at approximately 5900 rcf for 10 minutes. Discard the supernatant and resuspended in 30 mL of cold, sterile deionized water per tube. Centrifuge for another 10 minutes as before, and discard the supernatant.

3.3.3 Combine the contents of the tubes into multiples that will allow ease of centrifugation. Aseptically add 30 ML of cold, sterile deionized water to each tube and resuspend the spores.

3.3.4 Combine 58 mL of Hypaque™ with 42 mL of sterile, deionized water. Mix well. Add 12 mL of the Hypaque™ solution to a clean, sterile, 35-mL centrifuge tubes. Pipette the spore suspension, carefully layering it on top of the Hypaque™ solution. Centrifuge at approximately 5900 rcf for 30 minutes, using a swinging bucket rotor.

3.3.5 Pour off and discard the supernatant. Add 30 mL of cold, sterile deionized water to the pellet in each tube and resuspend the spores. Centrifuge at 5900 rcf for 15 minutes, using a fixed-angle rotor.

3.3.6 Discard the supernatant and resuspend the spores in 30 mL of cold, sterile deionized water. Wash the spores by centrifuging at 5900 rcf and resuspending twice more. Centrifuge again, discard the supernatant, and resuspend the spores in a 40% (v/v) ethanol solution. Store at 4ºC.

4.0 Waste Management

Dispose of all samples, solvents, reagents and laboratory wastes in accordance with the Laboratory's Waste Management Guidelines.

Appendix D

HEALTH AND SAFETY RESEARCH PROTOCOL

Prepared by: _____Date:_____

Approved by: _____Date:_____

U.S. ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF RESEARCH AND DEVELOPMENT
NATIONAL HOMELAND SECURITY RESEARCH CENTER

Purpose

To ensure adequate review of proposed occupational safety and health precautions, procedures, and techniques for the use, handling, storage, and waste disposal of hazardous agents utilized in research activities.

As the principal investigator, you should be most cognizant of the specific or potential hazards associated with agents upon which you are conducting investigations.

A hazardous agent, as defined by the AWBERC Health and Safety office, is one that has:

- An LD_{50} (oral, rat) <50 mg/kg body weight
- An inhalation LC50 toxicity (rat) of <2 mg/liter
- A dermal LD_{50} toxicity (rabbit) of <200 mg/kg
- One that causes carcinogenic effects (confirmed or suspect in humans and/or confirmed in animals)
- One that causes teratogenic or mutagenic effects (in humans or animals)
- Any infectious biological agent (as defined by CDC and/or NIH)
- Any explosive or violently reactive agent (shock sensitive, peroxide forming, and/or violently reactive with moisture/air)
- Is a sensitizing agent.

Note: Any material being studied because it is suspected of meeting any of the above criteria also requires a protocol.

Suggested online references:

- National Toxicology Program (http://ntp.niehs.nih.gov/ (Chemical Health and Safety Information)
- American Biological Safety Association (http://www.absa.org) (Provides a compendium on CDC, NIH, and other agent classifications for select agents)
- International Agency for Research on Cancer (http://www.iarc.fr)

- Online manufacturer MSDS sources can be selected through the “MSDS button,” available on the NRMRL Web page at http://ordnotes4.cin.epa.gov:9876/.

Additional references are available in the AWBERC Health and Safety Office, Room 270.

HEALTH AND SAFETY RESEARCH PROTOCOL
FOR HAZARDOUS AGENT RESEARCH
Andrew W. Breidenbach Environmental Research Center
Cincinnati, Ohio

Title of Study: Spore Adhesion to Pipe Material

Duration: 24 months

Principal Investigator (PI): Ben Packard

Laboratory, Division, Branch: NHSRC, WIPD

Location: Office: Room 326 Lab: AWBERC Biocontainment Suite or the T&E Organic Chemistry Laboratory

Phone: X7324

PI Signature Date

(Principal Investigator must be an EPA employee)

APPROVALS

Division Director Date

(Obtain signatures above prior to sending to the Safety Office)

Safety Officer Date

A. Personnel Authorized to Use Hazardous Agent(s):

1. Ben Packard

Are all personnel working with this study participants in the Medical Monitoring Program?

Yes X No If no, why not?

B. Location(s) where work will be conducted (include hazardous agent storage location).

All reagents will be stored or used in the AWBERC Biocontainment Suite as well as the T&E organic chemistry laboratory.

C. Brief description of study.

This project will deal with measuring the levels of spores that adhere to pipe materials. One part of the study will be to quantify the amount of biofilm on a surface. Methods that have been chosen to enumerate the biofilm are the determination of total protein and total carbohydrate in the biofilm matrix. The carbohydrate assay will require the use of phenol and concentrated sulfuric acid, and the protein quantitation requires the use of kits that are pre-made.

D. Describe in detail all potentially hazardous operations and duration.

Protein Carbohydrate Assays:

Phenol and sulfuric acid, which are required for the carbohydrate assay, will be stored and used in fume hoods and will be appropriately labeled and disposed of. The assay requires roughly .75 mL of concentrated sulfuric acid and 12 ul of phenol per .25 mL sample. Appropriate personal protection equipment (PPE), including lab coats, goggles, and 2–3 pairs of nitrile gloves, will be worn when conducting work with hazardous chemicals. When samples containing small amounts of phenol are taken out of the fume hoods, lids will be placed on plates that will be read on the plate reader in room 318. Protein quantitation kits will be stored in a refrigerator at 4ºC. These kits do not require the use of a chemical fume hood. However, lab coats, goggles, and nitrile gloves will be worn when using the kits.

Enumeration of Spores:

Standard plate counting using TSA media will be used for *B. globigii* spore enumeration.

Cleanup:

Phenol from the phenol-carbohydrate analysis will be disposed of in the drain. Due to the small quantities of phenol used per sample (~ 12 ul/sample), it can be washed down the drain. Samples containing sulfuric acid will be neutralized with sodium bicarbonate prior to discarding.

Test Duration: On-going.

E. Hazardous Agents (MSDS sheets are attached):

1. Name: Phenol, sulfuric acid, NanoOrange® protein kit reagents, Coomassie® protein test kit reagents

2. Quantity:

a. To be ordered: phenol, sulfuric acid, and protein quantitation kits

b. Maximum quantity needed for study: 40 mL of 90% phenol, 300 mL concentrated sulfuric acid, Coomassie® protein quantitation kit, and NanoOrange® protein quantitation kit. It may be the case that additional protein quantitation kits will be assessed prior to the study. If this is the case, the HASP will be updated and MSDS sheets for the additional reagents will be filed in the project safety file.

4. Method/Area of Storage:

Type of container: Phenol and sulfuric acid will be sorted in a chemical fume hood and will not leave the fume hood. Protein kits will be stored in a refrigerator at 4º C and will not leave the area.

Location: AWBERC Biocontainment Suite or T&E Organic chemistry laboratory

Refrigerated? Yes – protein kits.

5. Are special handling procedures required (e.g., weighing of stock in glove box or fume hood)? If so, explain.

Yes, all work with sulfuric acid and phenol will be conducted in a fume hood with the exception of reading the samples on the plate reader in AWBERC 318. Work with protein test kits can be conducted outside of the hood with lab coat, goggles, and gloves.

F. Toxicity: Phenol

Substance:

Phenol CAS #: (108-95-2)

Project Use (Quantity):

Phenol is used as a starch indicator in the Dubois phenol sulfuric acid test for carbohydrates. It causes the sample to turn yellowish and, therefore, allows the sample to be read and compared to a standard curve with glucose. Samples are read at 480 nm on a spectrophotometer. Approximately 40 mL of phenol will be needed to conduct the assays.

Emergency Overview:

Phenol is a hazardous organic compound that can be absorbed through the skin, ingested, or inhaled.

Route Exposure:

Very hazardous in case of skin contact (corrosive, irritant), of eye contact (irritant), of ingestion, of inhalation. Hazardous in case of skin contact (sensitizer, permeator). The amount of tissue damage depends on length of contact. Eye contact can result in corneal damage or blindness. Skin contact can produce inflammation and blistering. Inhalation of dust will produce irritation to gastro-intestinal or respiratory tract, characterized by burning, sneezing, and coughing. Severe over-exposure can produce lung damage, choking, unconsciousness, or death. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Physical Hazards

Environmental concerns	YES	
	NO	
Potential Electrical Hazards?		
	X	
Will Hazardous Waste Be Generated?	X	
Radioactive Materials Involved?		
	X	
Treatability Exemption Utilized?		X
Cryogenic Material Involved?		
	X	

LD_{50} or Other:

Typical MEL 2 ppm; typical OEL 1 ppm. Acute poisoning by ingestion, inhalation, or skin contact may lead to death. Phenol is readily absorbed through the skin. Highly toxic by inhalation. Corrosive - causes burns. Severe irritant.

G. Toxicity: Conc. Sulfuric Acid

Substance: 12 N Sulfuric Acid

CAS No.: 7664-93-9
Health Rating: 3 - Severe (Poison)
Flammability Rating: 0 - None
Reactivity Rating: 3 - Severe (Water Reactive)
Contact Rating: 4 - Extreme (Corrosive)

Project Use (Quantity):

Around 300 mL of sulfuric acid will be used to break down starches so that they can be quantified with phenol in the sulfuric acid carbohydrate test.

Emergency Overview:

Sulfuric acid is a corrosive liquid that can cause severe burns to all body tissue. It may be fatal if swallowed, contacted with skin, or inhaled. It can eventually cause cancer.

Route Exposure:

Inhalation:
Inhalation produces damaging effects on the mucous membranes and upper respiratory tract. Symptoms may include irritation of the nose and throat, and labored breathing. May cause lung edema, a medical emergency.

Ingestion:
Corrosive. Swallowing can cause severe burns of the mouth, throat, and stomach, leading to death. Can cause sore throat, vomiting, and diarrhea. Circulatory collapse with clammy skin, weak and rapid pulse, shallow respirations, and scanty urine may follow ingestion or skin contact. Circulatory shock is often the immediate cause of death.

Skin Contact:
Corrosive. Symptoms of redness, pain, and severe burn can occur. Circulatory collapse with clammy skin, weak and rapid pulse, shallow respirations, and scanty urine may follow skin contact or ingestion. Circulatory shock is often the immediate cause of death.

Eye Contact:
Corrosive. Contact can cause blurred vision, redness, pain and severe tissue burns. Can cause blindness.

First Aid Measures:

Inhalation:
Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Call a physician immediately.

Ingestion:
DO NOT INDUCE VOMITING. Give large quantities of water. Never give anything by mouth to an unconscious person. Call a physician immediately.

Skin Contact:
In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Wash clothing before reuse. Excess acid on skin can be neutralized with a 2% solution of bicarbonate of soda. Call a physician immediately.

Eye Contact:
Immediately flush eyes with gentle but large stream of water for at least 15 minutes, lifting lower and upper eyelids occasionally. Call a physician immediately.

Physical Hazards

	YES	NO
Environmental Concerns		
Potential Electrical Hazards?	X	
Will Hazardous Waste Be Generated?		X
Radioactive Materials Involved?		X
Treatability Exemption Utilized?		X
Cryogenic Material Involved?		X

LD_{50} or other :
Oral rat LD_{50}: 2140 mg/kg; inhalation rat LC50: 510 mg/m3/2H; standard Draize, eye rabbit, 250 ug (severe); investigated as a tumorigen, mutagen, reproductive effector.

Carcinogenicity:
The International Agency for Research on Cancer (IARC) has classified “strong inorganic acid mists containing sulfuric acid” as a known human carcinogen, (IARC category 1). This classification applies only to mists containing sulfuric acid and not to sulfuric acid or sulfuric acid solution.

H. Toxicity: NanoOrange® Protein Quantitation Kit

Substance:

Component A: dimethylsulfoxide (DMSO) (1 mL) CAS Number: 67-69-5

Project Use (Quantity):

DMSO is the active ingredient. Extremely small amounts are used (~3ul/ sample) so it does not pose a risk.

Emergency Overview:

Irritating to eyes, respiratory system, and skin. Readily absorbed through the skin.

Route Exposure:

See emergency overview.

First Aid Measures:

If eye or skin contact occurs, wash affected area with water for 15 minutes and seek medical advice. If inhaled, move individual to fresh air and seek medical advice. If swallowed, seek medical advice.

Physical Hazards:

	YES	NO
Environmental Concerns		X
Potential Electrical Hazards?	X	
Will Hazardous Waste Be Generated?		X
Radioactive Materials Involved?		X
Treatability Exemption Utilized?		X
Cryogenic Material Involved?		X

RTECS Number: PV6210000

Toxicity:

LD_{50}: 14.5 g/kg, oral, rat; LD_{50}: 8.2 g/kg, intraperitoneal, rat.
Irritation data: 500 mg/24H, Std. Draize, skin, rabbit, mild; 500 mg/24H, Std. Draize, eyes, rabbit, mild.

Health Hazards:

See Toxicity and Health Hazards Data above.

Carcinogenicity:

Not listed by NTP, IARC or OSHA.

I. Toxicity: Substance: Coomassie Protein Assay Reagents

Substance:

Ingredient Name	CAS No.
1) Phosphoric Acid	7664-38-2
2) Methanol	67-56-1

Project Use (Quantity):

Approximately .5 mL of Coomassie reagent is used per sample to determine the protein content of the sample. The kit comes with 500 mL of assay reagent. Reagent is added to samples in a microplate, which is then read in a microplate reader at 595 nm.

Emergency Overview:

Reagents contain material that cause damage to lungs, gastrointestinal tract, respiratory tract, skin, and eyes, if these areas are directly exposed to the reagent. Therefore, prolonged contact with the reagent should be avoided and the reagent should be kept in a sealed container when not in use. Reagent is slightly hazardous in case of skin contact (corrosive, irritant). Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Route of Exposure:

Absorbed through skin. Eye contact. Inhalation. Ingestion.

Effects and Symptoms:

Inhalation:
Slightly hazardous in case of inhalation (lung irritant, lung corrosive). Slightly hazardous in case of ingestion.

Ingestion:
Slightly hazardous in case of ingestion.

Skin Contact:

Slightly hazardous in case of skin contact (corrosive, irritant). Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Eye Contact:
Slightly hazardous in case of eye contact (irritant, corrosive).

Aggravating conditions:
Repeated or prolonged exposure is not known to aggravate medical condition.

First Aid Measures:

Inhalation:
If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion:
Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact:
In case of contact, immediately flush skin with plenty of water. Cover the irritated skin with an emollient. Remove contaminated clothing and shoes. Cold water may be used. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact:
Check for and remove any contact lenses. Immediately flush eyes with running water for at least 15 minutes, keeping eyelids open. Cold water may be used. Get medical attention if irritation occurs.

Notes to Physician: Not available.

Protection of first-aiders: Not available.

Physical Hazards:

	YES	NO
Environmental concerns		
Potential Electrical Hazards?	X	
Will Hazardous Waste Be Generated?		X
Radioactive Materials Involved?		X
Treatability Exemption Utilized?		X
Cryogenic Material Involved?		X

LD_{50} or other:

Acute oral toxicity (LD_{50}): 1530 mg/kg [Rat]. (Acid).
Acute dermal toxicity (LD_{50}): 2470 mg/kg [Rabbit]. (Acid).

Carcinogenicity:

Classified 4 (No evidence.) by NTP, None. by OSHA [Coomassie Plus® Protein Assay Reagent Component 1]. Classified 4 (No evidence.) by NTP, None by OSHA, None by NIOSH

J. Protective Equipment Required? Yes X No What type?

- For all chemical use, face, eye, and skin protection is required.
- Hand protection (gloves) is required when working around all chemicals.
- Laboratory coat is required when working around all chemicals.
- Respiratory protection is not required when chemicals; however, phenol and sulfuric acid will be used in a fume hood.

X Safety Glasses with splash protection
□ Splash Goggles
□ Face Shield
□ Other
□ Chemical(Butyl, Viton, □ Nitrile,_____)
X Nitrile
□ Cotton
□ Leather
□ Thermal (autoclaving)
□ Double gloves
X Lab Coat(tyvek, cotton)
□ Lab Apron
□ Jumpsuit
□ Other
□ Air Purifying-full face
□ Air Purifying-half mask
□ Surgical mask
□ Dust mask-not true respirator

K. Explain any special conditions:

Precautionary Procedures to Be Used (e.g., controlled access, covered work surfaces, etc.):

See section E for detailed precautionary protocol.

Type of hood? Standard chemical fume hood.

Emergency Procedures:

In the event of an accident or spill (loss of control):

Phenol will be absorbed with an absorbent and disposed of as a hazardous waste. Any manipulation such as pipetting phenol will be conducted in the fume hood. The only phenol that will be outside of the hood will be samples containing < 1 ug/ml per sample. Sulfuric acid will also be used only in the hood. In the event of a spill, sodium bicarbonate will be added to the sulfuric acid to neutralize it prior to cleaning up. Samples will have ~75% sulfuric acid and will be analyzed outside of the fume hood. Therefore, all required PPE will be worn when samples are transported and analyzed.

Reagents other than phenol will be absorbed using towels or absorbent and will be disposed of in the sink.

L. Hazardous waste disposal (all disposal will be through the Waste Management Program)

Type of waste:

Phenol: Pipettes, containers, and any other material that comes in contact with the phenol will be stored in a hazardous waste container for proper disposal.

Samples containing sulfuric acid will be neutralized in sodium bicarbonate prior to being disposed of in the sink.

Volume of waste:

One 500-mL container of contaminated phenol waste.

Unused stock (to be disposed of or kept):

Unused phenol will be saved for a period of time following the experiments and eventually will be disposed as a hazardous waste.
Unused protein quantitation kits will be saved for follow-on studies until they have reached their expiration date, when they will be disposed of through the waste management program.

Appendix B

Quality Assurance Project Plan (QAPP)

Evaluation of Bacterial Endospore Retention on Common Drinking Water Pipe Surfaces

Benjamin Packard
Water Infrastructure Protection Division
National Homeland Security Research Center
U.S. Environmental Protection Agency

Approvals:

Benjamin Packard, Principal Investigator/WIPD	Date
Kim R. Fox, Director/WIPD	Date
Eletha Brady-Roberts, Director of Quality Assurance/NHSRC	Date

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CHAPTER 6: DISTRIBUTION LIST

Kim Fox, Division Director, Water Infrastructure Protection Division, NHSRC
Hiba Ernst, Associate Division Director, Water Infrastructure Protection Division, NHSRC
Eletha Brady-Roberts, Director of Quality Assurance, NHSRC
Margaret Kupferle, Professor of Environmental Engineering, University of Cincinnati

CHAPTER 7: PROJECT DESCRIPTION AND OBJECTIVES

7.1 Background

In the past decade, especially following 9/11 and the anthrax mailings, the possibility of persistent biological agents being used to shut down critical infrastructure has taken on increasing concern. Persistent biological agents require extensive and lengthy cleanups once they are distributed into the environment, and therefore, they have the potential to disrupt public health and the economic vitality of an area. *Bacillus* spores are recalcitrant and are able to stay viable in water for two years even in the presence of increased levels of chlorine (Watson and Keir 1994; Maginnis, Brazis et al. 2001). In light of the multiple impacts intentional contamination would have on a community, it is important to assess how to accurately surface sample water infrastructure in order to determine the extent of contamination as well as the efficacy of the decontamination of the system. In addition to sampling, it is important to understand potential levels of adhesion of organisms to the internal surfaces.

7.2 Process Description

The U.S. Environmental Protection Agency's National Homeland Security Research Center (NHSRC) in Cincinnati will conduct a study to investigate *Bacillus* spore attachment to drinking water pipe coupons. Because of the many variables involved in conducting risk assessments and modeling contamination scenarios of drinking water infrastructure, data are needed that address the differences in adhesion of *Bacillus* spores to pipe material and the most suitable ways to surface sample the wetted surfaces. This study will provide data on these two aspects. The following tables list variables that will be studied:

Table 9. Proposed study variables

	Pipe material			Pipe material conditioning		
Variable	Unlined cast and ductile iron	Cement lined cast and ductile iron	PVC	Biofilm grown from Cincinnati tap water	Bare coupon (no biofilms)	Artificial biofilm
Reason for study	Internal pipe surface material will exhibit varying characteristics with respect to surface roughness, surface charge, and level of biofilm growth.			Biofilm is ubiquitous in drinking water distribution systems	Biofilm is patchy and may not be present on all surfaces	Artificial biofilm could be used in place for future research
System averages*	18%	50%	8%	Multiple factors influence biofilm growth on pipe	Multiple factors influence biofilm growth on pipe	No current way to quantify
Proposed variables	Unlined ductile iron, concrete-lined ductile iron, and PVC			Biofilm, artificial biofilm, and bare surface		

*Pipe material averages taken from the AWWA 2002 Water Stats Water Utility Distribution Survey (AWWA, 2002)

Table 10. Surface sampling methods

Sampling Method		
Brushing	**Scraping**	**Swabbing/Wiping**
Bristles may remove spores from porous surfaces more efficiently	Scraper may remove top layer of corrosion/biofilm more efficiently	Traditional and simpler method of microbiological surface sampling that does not require diluent

7.3 Project Objectives

The objectives of this project are to investigate the following:

1. Compare recovery efficiency of *Bacillus* endospores using three surface sampling techniques (brush, scrape, and wipe) on three pipe surfaces (unlined iron, cement-lined, and polyvinyl chloride[PVC]) with three pipe surface preparations (tap water biofilm, sterile drinking water, and artificial biofilm). The results from this part of the study will provide information on variability and recovery of spores off of wetted pipe material. This information may lead to conclusions on which sampling method to use given a particular combination of surface properties. These techniques may potentially be used to determine the extent of contamination and/or the efficacy of decontamination if pipe is exhumed or if wetted surfaces are available to sample.

2. Compare differences in adhesion of *Bacillus* endospores to pipe coupons with different surface preparations. Results from this part of the study will provide comparisons of the contamination potential of various pipe material and biofilm combinations. Additionally, the surfaces of biofilm and pipe material will be characterized using atomic force microscopy, scanning electron microscopy and surface profilometery to determine potential mechanisms of spore adhesion and reasons for differences in spore adhesion between pipe surfaces.

3. Evaluate the ability artificial biofilm to serve as a surrogate for naturally developed biofilms in spore retention experiments. Artificial biofilm models have been developed that mimic properties of natural biofilm. Thus, artificial biofilms may serve as a standardized test system for cross comparing and evaluating adhesion of contaminants to biofilm covered surfaces.

7.4 Project Organization

7.5 Personnel

Table 11. ***Table 2 Project participants and functions***

Name	Phone	Email	Function
Kim Fox	(513) 569-7820	fox.kim@epa.gov	Division Director
Hiba Ernst	(513) 569-7943	Ernst.hiba@epa.gov	Associate Division Director
Eletha Brady-Roberts	(515) 569-7662	roberts.eletha@epa.gov	QA Manager
Margaret Kupferle	(513) 556-3329	margaret.kupferle@uc.edu	U.C. Advisor for thesis
Ben Packard	(513) 569-7324	packard.benjamin@epa.gov	Technical Support/Principal Investigator

7.6 EPA Quality Assurance

Eletha Brady-Roberts, Director of Quality Assurance for NHSRC, will oversee project quality requirements for the EPA. As the Director of Quality Assurance, she serves in the Immediate Office of the Director of the NHSRC and is independent of project management.

7.7 Responsibilities of Project Participants

Ben Packard will be responsible for writing the QAPP and related Standard Operating Procedures (SOPs). He will also order supplies, maintain the laboratory, and ensure that the laboratory efforts are performed in a careful and controlled manner consistent with the QAPP. He will prepare media and reagents, perform the collection of data, and carry out analysis of that data. He will be in charge of modifying the study as required in order to obtain data that are conclusive and useful.

No specialized training or certification is required for any of the procedures involved in this project beyond the standard education and experience required for microbiology laboratory work.

7.8 Facility

The experiments will take place at EPA's research facilities in Cincinnati: the Andrew W. Breidenbach Environmental Research Center (AWBERC) and the Testing and Evaluation

Facility at MSD. Coupon characterization will be conducted at the University of Cincinnati Advanced Materials Characterization Laboratory and or contract labs.

CHAPTER 8: EXPERIMENTAL APPROACH

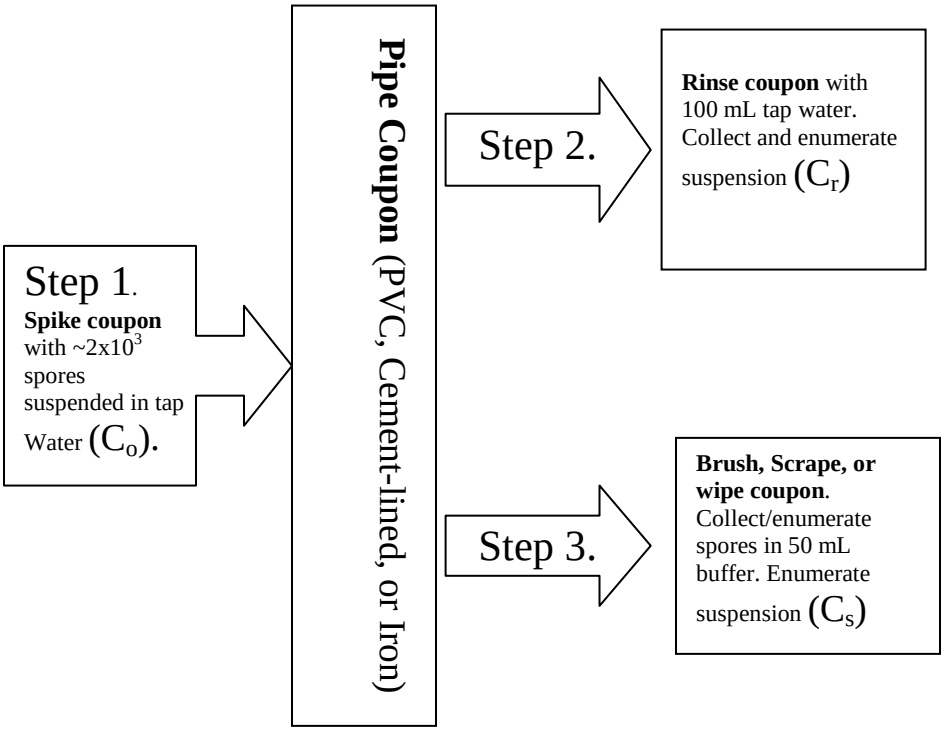
8.1 General Approach and Test Conditions

The experimental setup will consist of using Iron, cement-lined, and PVC coupons (3 cm by 13 cm) cut from new water mains. Coupons will be conditioned in three different ways before they are exposed to spores. A mass balance approach will be used to calculate the fraction of spores that adhere to the coupons and the fraction that are sampled from the coupons. Analysis of variance will be used to compare the different factors.

8.2 Experimental Approach

A mass balance approach will be used to calculate percent adhesion and percent recovery for each surface treatment or sampling method, respectively. The specific components of the mass balance are summarized in Figure 2.

Figure 12. Mass balance diagram showing samples taken to determine recovery efficiency



Equation 1. Recovery Efficiency

$$RE = \frac{C_s}{C_o - C_r} \times 100$$

Equation 2. Percent Adhesion

$$PA = \frac{C_o - C_r}{C_o} \times 100$$

8.2.1 Spore loading and coupon rinsing (Step 1)

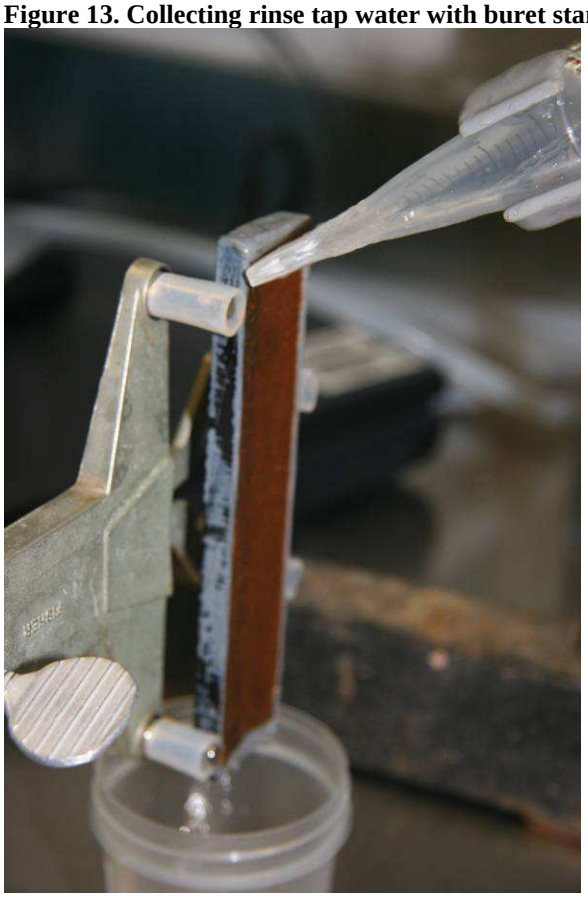
Approximately 10^3 spores suspended in 1 mL of dechlorinated tap water (step 1 figure 2) will be spiked onto the leveled coupon and left to sit for 20 min.

8.2.2 Coupon rinsing (Step 2)

At the end of the designated contact time as described in step 1, the contaminated side of each coupon will be rinsed so that all spores that did not adhere or were weakly adhered to the surface will be rinsed off. Rinsing will entail running 200 mL of water down the internal pipe face of the coupon as shown in figure 3. A sterile buret stand and a modified 30 mL syringe, connected to a peristaltic pump will be used as a nozzle to ensure that the water is dispersed across the entire coupon during rinsing. The rinse water will be collected in a sterile 250 mL sample container to be quantified. Parafilm will be wrapped

around the buret stand points of contact with the coupon and will be replaced prior to placing each new coupon in the stand.

Figure 13. Collecting rinse tap water with buret stand and modified 30 mL syringe



8.2.3 Spore sampling (Step 3)

Directly following rinsing, the contaminated surface of the coupon will be sampled by combinations of brushing with a tooth brush, scraping with either a cell scraper or sterile sampling spoon (depending on the amount of corrosion), and wiping. Sampling techniques were chosen on previous study data along with a an analysis of the most efficient way to sample pipe surfaces of different materials. Therefore, sampling will consist of a bulk removal step using either brushing or scraping followed by either wiping or rinsing using phosphate buffer with Tween 80. Wiping alone, will be tested as well since this is a common surface sampling technique and also is represents a convenient way to quickly sample an exhumed pipe.

Based on a previous study, brushing was found to have higher recovery efficiency than scraping with a cell scraper. Therefore, the iron and cement-lined coupons will be brushed due to their porous surfaces. In contrast, PVC will be scraped using a cell scraper

and/or sterile sampling spoon as the bulk removal step. The four sampling techniques to be tested are as follows:

- Scraping/brushing followed by wiping
- Scraping/brushing followed by rinsing
- Wiping followed by rinsing
- Wiping only

The technique for brushing and scraping will be to brush or scrape the coupon 5 times in the same manner and then wash the coupon and the brush/scraper with 100 mL of phosphate buffer with 0.01% Tween 80. This resulting spore suspension will be collected in a sterile petri dish and transferred to a 100 mL sample container. 4x4 Wipes will be wetted with 2 mL of phosphate buffer and Tween 80 before they are used. They will then be placed in a 100 mL sample container containing 100 mL, of the phosphate buffer and Tween 80 diluent. Recovery efficiency will be calculated as indicated in Figure 2 and Equation 1. Detailed sampling procedures for brushing, scraping, and wiping can be found in Appendix B.

8.3 Pipe Coupons

New pipe sections will be cut using a high-pressure water jet cutter. This method of cutting uses a very small high-pressure jet of water and abrasive material that can cut through up to 6 inches of steel while keeping the surface and the metal cool as it is cut. Dimensions for the cut are programmed into a computer that controls the machine.

Pipe material selection is based on the current statistics supplied by the American Water Works Association (AWWA 2002) for small, medium, and large drinking water utilities. This will include unlined ductile iron, concrete lined, and PVC pipe sections. The 39 cm^2 of sampling surface on a 3x13 cm^2 coupon should provide enough surface area to get representative samples of biofilm and spores.

8.3.1 Coupon Preparation

After cutting and prior to conditioning, the sides and backs of ductile iron and cement-lined ductile iron coupons will be sealed with epoxy so that they do not rust in the conditioning process. A bead of epoxy will also be applied around the internal surface of the coupon so that liquid can be contained within the pipe surface.

8.3.2 Coupon Cleaning

Prior to use, new coupons will be conditioned using the National Sanitation Foundation procedure for conditioning drinking water contact materials prior to determining the toxicity of the surface. This procedure is described in Appendix B Section 4.0 (ANSI/NSF 2000).

8.3.3 Pipe Coupon Surface Characterization

In order to understand the causes for the differences in recovery efficiency and spore adhesion, the following surface property tests may be conducted depending on available time and funding:

- Surface roughness with a profilometer and or atomic force microscope
- Coupon surface characterization of coupons with and without spores/biofilm with a Scanning Electron Microscope
- Biofilm chemical characterization using FT-IR to determine the functional groups in biofilm (including artificial biofilm)

Table 12.Coupon surface characterization experiments

	Surface roughness	Environmental SEM/AFM	Biofilm Chemistry
Measurement	Ra value	Surface topography	FT-IR
Location	UC Engineering College	UC Engineering College	EPA

Before experiments are carried out, an addendum including description of equipment, test-runs, methods, etc. will be submitted.

8.4 Coupon Conditioning

Since new pipe materials will be used in the experiments in this study, the pipe will need to be conditioned so that it exhibits the same properties as pipe that has been in service. Three procedures for preparing the coupons will be followed.

8.4.1 Bare surface with no artificial or natural biofilm

Coupons will be exposed to sterile drinking water for 24 hours prior to the experiments to condition the surfaces by placing coupons in 5 Liter buckets containing dechlorinated sterile tap water. Buckets of tap water will be autoclaved and coupons after having been cleaned (specified in Section 2.3.2) will be placed into the buckets containing the sterile tap water for 24 hours. This procedure will hydrate the coupon surfaces, as well as form a conditioning film formed from organic matter in the tap water on the coupon surfaces.

8.4.2 Drinking water conditioned coupons

Sterile coupons will be placed into an 8 inch PVC pipe cut in half length wise. Pipe coupons will then be placed in the pipe so that they are exposed to a continuous flow of Cincinnati tap water. Water will flow approximately 3 liters per minute. Coupons will be exposed in this manner for approximately 6 months. To measure biofilm growth over time, one polycarbonate coupon per month will be sampled. Prior to conducting

experiments, biofilm will be collected from each of the coupon types to determine the level of biofilm growth on each pipe material. Coupons will be brushed with a toothbrush to remove the biofilm which will be diluted and spread plated on R2A agar to determine the number of heterotrophic organisms (CFU/ cm^2) on the coupon surface. R2A plates will be bought from Remel (R01722) and QA samples as specified in section 4.0 table 9 will be taken.

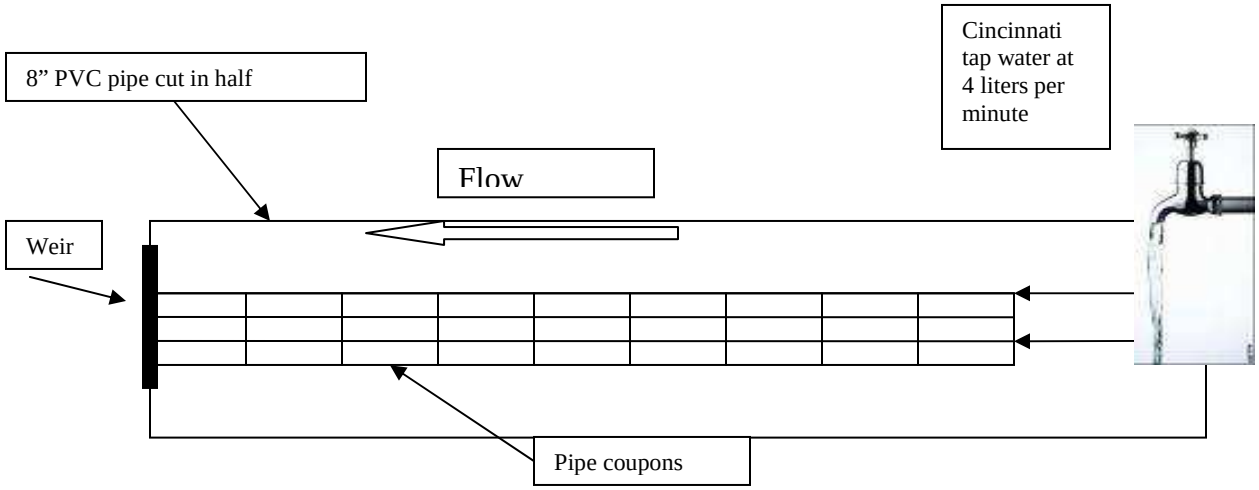


Figure 14. Flow box made from 8” PVC pipe section cut in half

8.4.3 Coupons with artificial biofilm

It has been shown that semi-porous agarose beads can be made and applied to a surface to produce an artificial biofilm that exhibits many of the properties of natural biofilms (Strathmann, Griebe et al. 2000). In order to determine whether this approach might be useful for follow-on research involving adhesion, sampling, and decontamination of water infrastructure, spore adhesion to coupons with artificial biofilm will be compared to coupons with drinking water biofilm. The ability to make artificial biofilm that exhibits the same properties as drinking water biofilm would alleviate the need to grow biofilm for future studies involving spore adhesion or disinfection. The artificial biofilm will be applied to coupons conditioned in tap water (same the bare surface conditioning procedure). However, after conditioning in tap water, coupons will be heated, and agarose beads will then be poured on the surface to form semi-porous biofilm as described in Appendix A. The artificial biofilm procedure is under development. Any changes to the procedure will be updated an addendum.

8.5 Bacterial Selection and Spore Development

Studies conducted to determine spore-forming *Bacillus* species that could be used as surrogates for *B. anthracis* have found that the mean *CT* (*C* is the concentration of chlorine in mg/liter, and *T* is the exposure time in minutes) values for *B. globigii* are

higher than the corresponding mean CT values for the three other surrogates and the virulent strain of *B. anthracis*, making *B. globigii* a good surrogate for persistence and disinfection studies (Rice, Adcock et al. 2005). *B. globigii* serves as a conservative surrogate for inactivation studies using chlorine. Thus, data generated from this project could be used in future studies involving disinfection. *B. globigii* spores will be grown as indicated in Appendix C.

8.5.1 Spore enumeration, viability, and accuracy checking

B. globigii spores will be enumerated using membrane filter procedures in section 9222A of Standard Methods for the Examination of Water and Wastewater (Clesceri 2005). The media for spore enumeration will be tripticase soy agar (TSA) plates as specified in Standard Method 9218 A. Aerobic Endospores. Pre-made plates will be bought from Remel (R01917) and QA samples as specified in section 4.0 table 9 will be taken. When dilutions of samples are needed, sterile phosphate buffer (0.05 M KH_2PO_4) with .01% Tween solution will be used as the diluent, as described in Appendix C. Colony forming units (CFU) will be counted to approximate the numbers of viable spores sampled from the coupon. Percent recovery will be calculated as described in section 2.5. It was determined from preliminary studies that 2 mL of approximately 10^3 spores will be used as a spiking spore suspension. This concentration was established from preliminary study results with various concentrations. The spore suspension concentration that provided the most reliable recoveries using membrane filtration at the lowest concentration was the 10^3 suspension. Contact time was based off of stokes equation calculation for gravity settling of a sphere the size of a spore made of protein.

8.5.2 Water quality monitoring

The following parameters will be measured in all tap water used for spore suspensions or rinse water before and after the experiments.

- pH
- Conductivity
- Temperature

8.6 Experimental Design

The objectives of the study, given in section
As was introduced in section 1.3, the primary objectives of the study were to:

1. Determine/compare recovery efficiency of Bacillus endospores
2. Determine/compare Bacillus endospore adhesion

Therefore, the specific research hypothesis of the study includes the following:

Recovery Efficiency

H01: Recovery efficiency of Bacillus endospores is the same across sampling techniques given combinations of pipe materials and surface preparations.

H02: For a given sampling technique, the recovery efficiency of Bacillus endospores is the same across combinations of pipe materials and surface preparations.

H03: For a given sampling technique and pipe material, the recovery efficiency of Bacillus endospores is the same across surface preparations.

H04: For a given pipe material, the recovery efficiency of Bacillus endospores is the same across surface preparations and sampling techniques.

Adhesion

H01: Adhesion of Bacillus endospores is the same across pipe materials and surface preparations

H02: For a given pipe material, spore adhesion is the same across surface preparations.

Experimental Design

Pipes of three different materials (iron, cement-lined, and PVC) will be selected at random and 90 coupons from each pipe material will be cut out. To reduce bias, coupons will be randomly allocated to the treatment groups. Additionally, sampling techniques (brushing, scraping) will be done in a random order by assigning the coupons random numbers. The laboratory and conditions will be kept as identical in every way apart from the applied treatments. Coupons will be assigned random numbers for each pipe material from which batches of 30 will be assigned to the different surface preparations. After sorting the random numbers, the coupons that correspond to the first 30 random numbers (ranks between 1 and 30) can be assigned to the first surface preparation, the second batch of 30 coupons (ranks between 21 and 60) to the second surface preparation, and the third set of coupons (ranks between 61 and 90) to the last surface preparation.

Sample Size and Power

The level of significance, α, defines the probability level that is too low to warrant support of the tested hypothesis. This rejection is equivalent to supporting one of the possible alternative hypotheses that is not contradicted by the data (e.g., the mean concentrations are different across sampling methods).

If the decision rules do not reject H_o, when it is in fact false, this also leads to an erroneous decision. This kind of error is known as a Type II error of failing to reject the tested hypothesis when it is false. The potential magnitude of a Type II error depends in part upon the level of significance of the test, and in part upon which of the alternative hypotheses the data actually supports. Associated with each possible alternative hypothesis is a different probability of a Type II error.

The current sample size for each treatment combination is 7. With this sample size and assuming the values obtained from the Phase I analysis it is possible to detect a difference of 35% recovery efficiency between two treatments (e.g., combination of pipe type, sampling method, and surface preparation).

8.6.1 Treatment Design for Recovery Efficiency Experiments

The treatment design for using the sampling recovery data includes 12 treatment combinations of sampling technique (brush/scrape-wipe, brush/scrape-rinse, wipe-rinse, and wipe), surface preparation (tap water biofilm, no biofilm, and artificial biofilm), and pipe material (Iron, cement-lined, and PVC). Factors to be compared will be pipe material, sampling technique, and coupon surface conditioning (4 x 3 x 3) as shown in the following table:

Table 13. Sampling Experiments

	PVC				Cement-lined				Iron		
	Scrape/Wipe	Scrape/Rinse	Wipe/Rinse	Wipe	Brush/Wipe	Brush /Rinse	Wipe /Rinse	Wipe	Brush/Wipe	Brush/Rinse	W
DW Biofilm	7	7	7	7	7	7	7	7	7	7	
Bare	7	7	7	7	7	7	7	7	7	7	
Artificial Biofilm	7	7	7	7	7	7	7	7	7	7	

8.6.2 Treatment Design for Spore Adhesion Experiments

Treatment design for adhesion data includes six treatment combinations of surface preparation (tap water biofilm, no biofilm, and artificial biofilm) and pipe material (Iron, Cement-lined, and PVC). Factors to be compared will be pipe material and coupon surface conditioning (3 x 3) as shown in the following table:

Table 14. Adhesion Experiments

	DW Biofilm	Bare	Artificial Biofilm
Iron	30	30	30
Cement-lined	30	30	30
PVC	30	30	30

8.7 Data Analysis

Statistical analysis will consist of evaluating whether there is a meaningful difference between spore recoveries or spore adhesion for the different treatments with a 95% confidence interval. Spore sampling recovery data will compare mean values using a three way ANOVA for recovery efficiency data and a two way ANOVA for adhesion data. Additionally, tests will be conducted to determine which surface characteristics influence adhesion the most by characterizing the coupon surface properties described above and correlating the values with the values gained through the spore adhesion experiments.

Unequal variance across treatments

The usual Analysis of Variance (ANOVA) (using PROC GLM in SAS) requires that certain assumptions be met if the statistical tests are to be valid. One of the assumptions is that the errors (residuals) all come from the same (Normal) distribution. Thus we have to test not only for normality, but we must also ensure (i.e., test) that the variances are homogeneous. The statistical test for homogeneity of variance is due to Bartlett and is a modification of the Neyman-Pearson likelihood ratio test.

CHAPTER 9: SAMPLING AND MEASUREMENT METHODS

The purpose of sampling using brushes, scrapers, and wipes will be to characterize different types of sampling methods and match them with pipe materials to determine the most consistent and effective sampling procedures. Additionally, precision will be determined for the different factors and compared.

9.1 Sampling Spores from Coupons

Specific procedures for brushing, scraping, and wiping as outlined in Appendix B will be tested to determine spore recovery. If, during the course of the experiments, procedures in Appendix B need to be altered the procedures will be revised accordingly. The following table summarizes the spore enumeration samples that will be taken for each coupon:

Table 15. Samples taken for each coupon to enumerate spores

Sample	Description	Reason for sample	Number of samples
Coupon rinse sample	Following inoculation, coupons will be rinsed with 100 mL of tap water to remove spores that have not adhered to the surface.	Quantify the number of spores that do not adhere to the coupon.	1
Coupon brush/scrape/wipe sample	Following rinsing, internal pipe surface of the coupon will be sampled using one of the three sampling techniques.	Quantify the number of spores removed with the toothbrush, scraper, or wipe.	1

Each sample will be spread plated on TSA agar plates in triplicate.

9.1.1 Steady-state Conditions

All glassware, solutions, and media will be brought to the target temperatures prior to use in the experiments. All incubators and refrigerators will be brought to 35 ± 0.5º C and 4 ± 2º C 1 hour before being used. The same materials (brushes, vials, stock buffer solutions, media, reagents, and pipettes) will be used for each series of experiments to ensure that there is no variability.

Glassware, stir bars, graduated cylinders will be autoclaved. Equipment that cannot be sterilized in an autoclave will be submerged in a 1:1000 bleach solution (i.e., Clorox®) for 1 hour and rinsed three times with Milli-Q® treated water. Glassware and stir bars will be baked in a dry oven at 180ºC for three hours.

Procedures for using tap water are outlined in Appendix C. In addition, CFU will be assessed with respect to the variables listed on Table 10.

All glassware and plastic sample containers containing samples to be enumerated will be spiked with a Tween 80 solution to prevent microorganisms from sticking to them.

9.1.2 Site Preparation

All analyses will be performed on a bench top that has been disinfected with a 10% Clorox solution that has been adjusted to a pH of 6.0 to 6.5. All microbiological analysis conducted at the Test and Evaluation facility will be conducted in a clean bench in the high bay area, or in the BSL-2 laboratory.

9.1.3 Cross-contamination

All sample containers will be labeled appropriately with name of sample, sample number, and date. All dilution blanks and spread plates will be labeled with sample number, dilution, and date. Sterile disposable pipette tips will be used and discarded immediately after use. Spreading rods will be dipped in ethanol and burned in a flame after each plate is spread. Brushes and scrapers, and wipes will be discarded after being used. Wipes will be autoclaved before use and all solutions and glassware used in sampling biofilm or spores will be sterilized prior to use.

9.2 Representative Samples

Biofilm is variable within a distribution system and the amount of biofilm may depend on multiple factors. Coupons exposed to tap water to grow biofilm will be randomized in time so that coupons will be taken from the flow channel randomly from different parts of the device.

9.3 Sample Identification

Each sample tube, each dilution tube, and each spread plate will be pre-labeled with exposure time, pipe material, dilution, concentration, and date. The laboratory notebook and bench sheets will be filled out as the tests progress and data will be recorded as soon as they are read. Plate counts recorded onto bench sheets will be recorded in the laboratory notebook will be entered into an Excel file following the experiments. Data in the Excel file will be double-checked with bench-sheet data to ensure that no transcription errors were made.

9.4 Testing and Measurement Protocols

The following charts summarize the measurement protocols to be used for biofilm and spore enumeration as well as for water parameter sampling:

Table 16. Sample Measurements

Parameter	Analytical method	Sampling procedure	Sample size/ container	Replicates	Preservation and storage	Maximum holding time
Heterotrophic Plate Count	*Standard Method 9215A.*	Scraped from 38 cm^2 coupon into large Petri dish	100 mL sample container/ .1 mL spreadplate	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Spread plating	*Standard Method 9215C*	Scraped from 39 cm^2 into 120 mL coliform sample bottle	~.1 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours
Membrane Filtration for aerobic endospores	*Standard method 9218.*	Scraped from 39 cm^2 into 120 mL coliform sample bottle	3-5 mL	3	Initiate analysis immediately, or store at 4ºC.	24 hours

Table 17. Water Quality Samples

Parameter	Analytical method	Reference	Sample size/ container	Preservation and storage	Maximum holding time
pH	pH-meter	*Standard Methods* 1992	In situ	NA	NA
Conductivity	Extech handheld conductivity and temperature meter	*Standard Methods 2510 B*	In situ	NA	NA
Temperature	Extech handheld conductivity and temperature meter	*Standard Methods* 2550	In situ	NA	NA
Total and free chlorine	Hach DR 2400 Spectrophotometer	*Standard Methods 4500-CL G.*	50 mL beaker	NA	NA

During the course of the experiments, if one of the methods listed in the preceding charts proves to be unusable, another method may be used. If this is the case, new protocols will be chosen and the QAPP and will be updated accordingly.

Media	Measurement	QA/QC Check	Acceptance Criteria	Corrective Action	Frequency
Water Parameters		Check Standard	80-120% recovery	Retake samples	
	pH	Check against pH 7 buffer	± 0.2 pH units	Use different DO meter	Every sampling event
	Alkalinity	Negative control samples	6.9<pH<7.1	Recalibrate meter and recheck	When time and resources permig
	Conductivity	Check solutions of 200 mS and 2.00 mS	Reading ± 10% of expected value	Adjust meter	During Phase 2 experiments
	Free and total chlorine	Check tap water fresh from the system	Reading ± 10% of expected value (~1 mg/L)	Calibrate spectrophotometer/ replace reagents	Weekly when coupons are exposed to drinking water
	Temperature	Check thermometer against a calibrated thermometer	±0.5ºC	Note discrepancies	Weekly and during every sampling event
Coupon	Spore Enumeration	TSA Blank: Incubate plates	No observed growth	Remake plates	Every spore sampling event
		Spore Viability and quantitation: Positive Control plate spike (Known quantity of spores spiked onto TSA)	100-200 CFU/plate	Check spore suspension and dilution procedures	Every spore sampling event
		Coupon check: Process coupon without adding spores to determine if there is contamination on the coupon. (this is combined with matrix spike)	No observed growth	Determine potential sources of contamination, sterilize all surfaces and equipment and process a second coupon.	Two times for each surface preparation pipe material combination
		Buffer check: Plate buffer solution on TSA to determine sterility	No observed growth	Remake buffer/autoclave all glassware	Every spore sampling event
		Tap water check: Plate tap water used for growing biofilm, and rinsing/spiking coupons	No observed *B. globigii* growth	Sample tap water from tank a second time and autoclave all glassware	Every spore sampling event
		Matrix spike: Scrape biofilm from iron, cement-lined, and PVC. Spike each with 1 mL of spore suspension to determine impact of corrosion on spore enumeration.	100-200 CFU/plate	Compare to counts without corrosion and note differences	Two replicates for each phase.
		Membrane Filter Sterility check: Sterile PBDW with .01% Tween added to MF UV sterilized funnel and sterile filter.	No observed *B. globigii* growth	Resterilize funnel again, ensure that filters are sterile, clean funnels to ensure there is no rust/corrosion on surfaces.	One check for every 10 samples.
	Biofilm HPC	Buffer blank: Plate buffer solution on R2A plates	No observed growth	Remake buffer	Every biofilm sampling event
		R2A Blank: Incubate plates	No observed growth	Remake plates	Every biofilm sampling event

CHAPTER 10: QA Objectives

10.1 Quality Assurance/Quality Control

All analytical samples taken will include positive and negative controls to ensure that the samples that are analyzed are accurately defined. No fewer than three replicates will be taken to get representative samples. Samples that are outside of the limits of detection, or that do not meet the acceptance criteria required for the method, will be noted and checked for possible errors contributing to the result. Where possible, the tests will be repeated. Table 9 summarizes the QA samples to be taken.

Table 18. QA/QC Criteria

10.2 Equipment

SOPs for the various assays will be kept in the laboratory along with the lab notebook. The laboratory notebook will provide a log of procedures for each experiment. Where possible, the appropriate SOP will be referred to unless deviated from in the experiment.

Equipment such as the pH meters will be used in accordance with the manufacturer's manuals for those pieces of equipment. Larger equipment (refrigerators, autoclaves) will be operated using the standard SOPs outlined in the facility SOP manual. Laboratory equipment and instrumentation will be checked for accuracy as directed in Standard Methods 9020 B. The following chart summarizes data requirements for equipment:

Table 19. Data Requirements

Data Quality Acceptable If…	Corrective Action If Unacceptable
All equipment, sensor, and meter calibrations are current.	Out-of-date calibrations will be corrected by recalibration or replacement of item and the analysis redone.
Reagents used are not past expiration date. Reagent grade chemicals are used. Microbiological grade agar used. Traceable standards. Negative controls are used to test for sterility.	Expired reagents will be replaced and the analysis redone. Reject chemicals/agar that is not proper grade.
Refrigerators, autoclaves, and incubators are operating at the required temperature.	If temperatures were not maintained within the required limits of the test, the equipment will be fixed or calibrated and the analysis will be redone.

The PI will ensure that equipment requiring calibration and monitoring is maintained in accordance with the manufacturer's guidelines and EPA requirements. The following chart summarizes calibration, use, and maintenance for the equipment to be used:

Table 20. ***Calibration, Use, and Maintenance of Equipment***

Equipment	QA/QC	Frequency	Acceptable Criteria	Corrective Measures
Centrifuges	EPA centrifuges are under a Preventative Maintenance Agreement (PMA) and undergo an annual PM.	Quarterly or as described	Described in instrument manual or SOP	Described in instrument manual or SOP: All non-conformances are corrected before the instrument is put back to use.
Autoclaves	Autoclaves use tape with each batch that contains the date, time, length of cycle, and contents of the cycle. Autoclaves are under a PMA and undergo a quarterly PM.			
NanoPure water purification system	Check conductivity			
Temperature controlled devices	All water baths, refrigerators, freezers, etc. will be checked prior to and after use.			
Balances	Balances are calibrated monthly and annually serviced through the PMA.			
pH and dissolved oxygen meters	pH meters are calibrated daily in buffer.			
Micropipettes	Micropipettes are calibrated annually.	Annually or as described		
Tracking and assessment of media, reagents and supplies	All medium is purchased from a nationally recognized supplier. Negative and positive controls are included in each batch. Lot, date of receipt, and expiration dates are all recorded in the laboratory logbook for reagents and media.	As needed or when new chemicals are received	Ensure critical information is recorded.	Document if information is available; qualify any results that are suspect based on uncertainties with expiration dates.

10.3 Data Documentation

All documentation of testing and results shall be kept in a project-specific file. The laboratory notebook or laboratory bench sheets bench sheets will contain the following for each experiment:

- Date of test run
- Data from water parameter analysis (pH, temperature, total and free chlorine, etc.)
- Counts from plates
- Counts for negative control plates
- Conditions/factors that are outside of the regular parameters of the standard operating procedure.

Data will be entered into a Microsoft Excel Spreadsheet. In addition to laboratory bench sheets, procedures, protocols, and observations will be recorded directly in a laboratory notebook, which will be present during all experiments.

10.4 Data Review

The Quality Manager will review test records to identify and resolve any inconsistencies. The person performing the review will initial the data copy and date it. All the hard copies will be kept in a binder and all electronic data will saved on CDs.

10.5 Data Reporting / Reduction and Validation

- Free and total chlorine, TOC values will be reported in mg/L. This value is read directly from the spectrophotometer or TOC analyzer.

- Microbial enumeration will be reported as colony forming units per milliliter (CFU/mL) for spread plates and membrane filters will be reported will be the average value of the CFU/mL from triplicate plates. Counts will also be expressed in CFU/mL for suspensions and samples, and CFU/cm^2 for numbers of spores adhered to surfaces.

- pH is read directly from the pH meter and is defined at the negative log10 of the hydrogen ion concentration.

- Conductivity will be read directly from the conductivity meter and will be reported in microSiemens/cm (µS/cm)

- Temperature is read in degrees Celsius (^oC) directly from a thermometer.

10.6 Deliverables

Reports will be prepared summarizing the results of the study. If the results from this study are compiled for publication, the report will proceed through NHSRC management review.

10.7 Data Validation

Data and calculations will be double checked to ensure that data were correctly recorded and transferred. All EPA data records management will be filed. Data collected in the lab will be recorded on laboratory bench sheets and transferred to Excel files as soon as possible.

CHAPTER 11: References

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Strathmann, M., T. Griebe, et al. (2000). "Artificial biofilm model- A useful tool for biofilm research." Applied Microbiology and Biotechnology **54**(2): 231-237.

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Appendix A: ARTIFICIAL BIOFILM

0.0 Scope and Application

The procedures listed in this appendix describes the procedure for making and applying artificial biofilm. Preparation of superporous agarose beads was adapted from (Strathmann, Griebe et al. 2000).

1.0 Health and Safety Warnings

- Observe all safety procedures discussed in the QAPP HASP, the AWBERC Chemical Hygeine plan in addition to any site-specific safety considerations.
- Report all accidents to the ORD Safety, Health and Environmental Management Office.
- Ensure that all work with Cyclohexane occurs in a well ventilated area away from heat, sparks, and Flames.

2.0 Equipment

2.1 Equipment

- Water bath
- Hot plate (2)
- Stir bar
- Thermometer (3)
- 1500 mL beaker (1)
- 250 mL beakers (1)
- 1000 mL beaker (1)
- Timer
- Mixer

2.2 Chemicals

- Agarose powder (Ameresco Agarose SFR™)
- DI water
- Tween 80
- Cyclohexane
- Span 85

3.0 Double Emulsification Procedure

1. Prepare 100 mL of a solution of agarose and water (4% wt/vol) in a 1500 mL Beaker.
2. Heat suspension to 95-100ºC on a hot plate (high heat) with magnetic stir bar rapidly mixing to ensure that agarose does not burn onto bottom of beaker.
3. Allow agarose to cool while stirring mL beaker. (This takes ~15 min and must be watched.)
4. Pre-warm a mixture of 3 mL Tween 80 and 40 ml cyclohexane and place in water bath #2 that is set at 50ºC.
5. Prepare a solution of 12 mL of Span 85 in 300 mL cyclohexane (#2). Warm solution to 47º C and pour into the mixture.
6. Turn hotplate heater to low temperature (~70º-80º) add cyclohexane/Tween mixture to agarose.
7. Start warming cyclohexane solution #2 to 50 º C.
8. Emulsify by stirring at 1200 rpm for 5 min while remaining in water bath (emulsion 1)
9. Add Emulsion #2, turn off hotplate and stir at 500 RPM for 10 min while agarose solution held at 50ºC. (emulsion 2)
10. Remove the beaker containing the agarose from water bath #2 and cool solution to room temperature while continuing to mix the solution. As the temperature decreases, the agarose solidifies into super-porous spherical particles.
11. Decant solvent and wash beads twice with DI water. Beads may take up to ½ hour to settle.
12. Store beads in sterile tap water and decant water prior to use. Beads can be stored for up to 1 week without swelling.
13. Prior to applying beads to coupons, fill a sterile beaker to the 40 mL mark with beads. Fill the beaker to 50 mL with sterile tap water and mix the suspension.

4.0 Applying Agarose Beads to Pipe Coupons

1. Ensure that coupons to be used have been sterilized and have been placed in sterile tap water for 24 hours as described in section 2.3.2
2. Allow coupons to dry by placing them in a sterile container until all moisture on the coupon surface has evaporated.
3. Prepare a solution of DI water and agarose powder (2% wt to volume).
4. Heat agarose solution to 90 ºC while mixing then cool to 50 ºC.
5. Using a sterile laboratory spoon, apply one spoon-full onto the beads.
6. Allow 5 minutes for the beads to settle and adhere to the coupon surface.

5.0 Waste Management

Dispose of all samples, solvents, reagents and laboratory wastes in accordance with the Laboratory's Waste Management Guidelines. Ensure that the cyclohexane is disposed of properly in a properly marked container.

Appendix B: SPORE SAMPLING PROCEDURES

11.0 Scope and Application

Techniques for sampling microorganisms from surfaces vary widely, and therefore, there is no universal or standardized way to sample surfaces. Problems with removing bacteria occur especially when the surface is porous. This procedure describes the plan to evaluate surface sampling methods to determine which is the most effective. There are several ways in which microorganisms can be extracted from a surface. It has been shown that procedures for collection of *Bacillus* spores using swabs, wipes, and vacuum socks on nonporous surfaces typically tends to underestimate the actual number of spores on the surface (Rose, Jensen et al. 2004). This is most likely to be true in the case of sampling from a porous pipe surface such as concrete or corroded iron. This evaluation will determine the relative sampling efficiencies and methods of spore removal in order to determine which method will work the best.

12.0 Summary of Method

This Appendix will explain procedures for physically removing spores from a pipe coupon cut from a water main. The procedures to be assessed are the following:

- Scraping the corrosion off the coupon using a cell scraper.
- Brushing the coupon with a brush
- Wiping the coupon with a sterile wipe

13.0 Health and Safety Warnings

The analyst must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

14.0 Cautions

14.1 Reagent standards must be prepared fresh on the day of the analysis.

14.2 Determination must be made within 48 hours of collection and sample stored at 4º C.

14.3 TSA plates must be read no more than 24 hours after the addition of spore suspensions.

14.4 Heat shocking of spores should last no longer than 10 min over 90º C

15.0 Preparing Coupons and Spore Suspensions

15.1 Coupons cut from new sections of pipe will undergo the following conditioning National Sanitation Foundation conditioning process prior to use:

15.1.1 Scrub coupons in tap water, using a test tube brush to remove any miscellaneous debris.

15.1.2 Using a spray bottle, spray the coupons and racks used to hold coupons with 200 mg/L sodium hypochlorite solution, coating all surfaces of the coupon.

15.1.3 Let the coupons and racks stand for at least 30 minutes, and then rinse with tap water.

15.1.4 Place coupons in racks, again rinse with tap water, and then rinse with deionized water.

16.0 Preparing for Coupon Spiking and Sampling

16.1 Ensure that the following items are turned on and are calibrated and the lab has been disinfected:

- Autoclave or steam sterilizer capable of achieving 121ºC (15 l)
- Hot water bath set to ~90º C
- Incubator set to 35ºC
- Disinfect all bench tops, and biosafety cabinets with .01% bleach solution
- Filter Manifold -Fisher cat # xx2504735
- vacuum gauge and connectors for manifold vacuum line (such as Cole Parmer gauge 07380-62, connector kit, 07395-20 and bushing 08539-83)

16.2 Assemble the following on a sterile bench

- Paper towels
- 100 mL sterile sample containers
- 250 mL sterile sample containers
- Rinse bottles filled with 50 mL of a PBDW and .01% Tween solution.
- Toothbrushes/cell scrapers
- Pipette, adjustable – 1–200 uL, 100–1000 uL with sterile pipette tips
- 10 mL sterile disposable pipettes
- 50 mL sterile disposable pipettes

- Vortex mixer
- 10^3 CFU/mL spore suspension – Spore suspension to inoculate coupon
- Certified timer
- Peristaltic pump. Ensure that pump is calibrated to ~ 100 mL /min
- Sterile forceps
- .45 µm membrane filters.

16.3 Ensure that the following are standing by on the bench prior to collecting coupon samples:

- Paper towels
- 10% bleach solution
- Cell spreaders and alcohol flaming equipment
- 150 mm TSA agar 100 mm petri dishes
- Blanks filled with 9 mL of PBDW.
- Pipette, adjustable – 1–200 uL, 100–1000 uL with sterile pipette tips
- Vortex mixer
- Sterile 500 mL beaker or flask
- 5 sterile 50 mL test tubes filled with PBDW and .01% Tween 80
- Sterile scrapers, wipes, and toothbrushes

Make sure that plates and dilution blanks are labeled and staged in the order that they will be used. Prior to sampling and spiking coupons, make sure that the water bath is on and set to 90º C, the incubator is set to 35ºC and the autoclave is on. Sterilize all surfaces that are to be used and assemble equipment and consumable items described above on the bench or biosafety cabinet. Set up coupon racks, sample bottles, ring stands and peristaltic pump to be used for spore spiking, rinsing, and removal in the biosafety cabinet or clean bench. Take a 1L water sample in a sterile beaker, measurre pH, conductivity, and total, and free chlorine. This will be used as rinse water and spore suspension to be used for spiking the coupons.

17.0 Procedure

17.1 Spore suspension

Defrost a 1.5 mL Eppendorf tube containing prepared concentrated spore suspension. Add to 100 mL sample container with 100 mL of tap water. Pour the spore suspension in the Eppendorf tube into the sample container. This is the spore suspension to be used to spike coupons. Label the bottle accordingly.

17.2 Coupon Collection

To collect coupons from trough, place 500 mL of tap water in a sterile beaker. Place coupon from the trough in the beaker and bring the sample back to the lab or clean bench. Place the coupon with the internal surface facing up on a sterile 15 inch petri dish.

Coupons with artificial biofilm and coupons with no biofilm should kept in sealed containers prepared the day prior. As needed, coupons should be taken out of the containers and placed in a sterile Petri dish with the internal pipe surrface facing up.

17.3 Coupon Inoculation

Spike the surface with 2 mL of the suspension in a 2 inch area in the middle of the coupon. Once the entire surface of the coupon is covered, set timer for 30 minutes and activate a countdown timer.

17.4 Coupon Rinsing

After the coupon has been inoculated with the spore suspension, pick up the coupon and transfer any residual suspension on the surface into the rinse sample container. Attach the coupon to the buret stand and place sterile rinsing nozzle next to coupon as shown in section 2.5. Using tap water from a peristaltic pump rinse the coupon with 200 mL of tap water and collect rinse water in the rinse container.

17.5 Spore Sampling

Sampling for spores will consist of comparing different combinations of brushing or scraping, wiping, and rinsing. Brushing and scraping will serve as a bulk removal step where large quantities of corrosion and biofilm are removed efficiently from the surface. Wiping and rinsing will aid in removing material left on the coupon surface that may be left after the majority of the bulk biofilm has been removed. The following sampling techniques will be tested for each type of coupon.

17.5.1 Brushing

- Brush the coupon using downward and diagonal strokes ensuring that all of the material on the coupon goes into the sample container.
- Between brushing, collecting the rinse in the petri dish. Complete this process 4 times.

- Transfer the PBDW to a sterile 100 mL sample container. Pour the remaining PBDW that is in the rinse bottle into the petri dish to rinse the dish and pour the rinse into the sample container.

17.5.2 Scraping

- Using a sterile disposable cell scraper or sterile spoon, scrape the top layer of corrosion and biofilm into a sterile 15 cm

petri dish. Ensure that all of the material that is scraped off the coupon surface is deposited into the petri dish. Scrape the coupon from both directions horizontally so that the biofilm/spores are scraped to the center of the coupon. Then lift the corrosion/biofilm off of the pipe coupon and place on the sample container.

- Transfer the PBDW to a sterile 100 mL sample container. Pour the remaining PBDW remaining in the rinse bottle into the petri dish to rinse the dish and pour the rinse into the sample container.

17.5.3 Wiping

- Prior to using gauze wipes, remove them from original packaging and wrap each gauze wipe section in aluminum foil and sterilize in an autoclave at 121^{o}C for 15 minutes
- Don sterile gloves
- Remove wipe and with one hand: Wipe back and forth to ensure that the entire surface area is covered. Fold the wipe over and wipe back and forth to cover the entire coupon surface.
- Fold the gauze with exposed side in and places gauze sponge pad in appropriate sterile sample container.
- For coupons with no bulk removal step (brushing/scraping), two wipes will be used; the first will remove the bulk material on the surface, and the second will be used to clean up any remaining material on the pipe surface.

17.5.4 Rinse

- Using a rinse bottle containing 100 mL of phosphate buffer containing .01% Tween 80, rinse the coupon surface thoroughly to wash all components of the sample into the sterile cup.

17.6 Sample Processing for Brush Scrape and Wipe Samples

17.6.1 Ensure that the plates are at room temperature and are properly labeled. The following is the plate labeling scheme:

- Pipe material: (Iron – I; Concrete – C; PVC – P)
- Action: (Scrape – S; Brush – B; Wipe-W) (Rinse – R)
- Dilution: (0; -1; -2; etc.)
- Prior to pipeting suspensions onto plates, double check that all material has been legibly labeled.

17.6.2 Following heat treatment, vortex the tubes and sample bottles solutions for 30 seconds. Make serial dilutions as required. Make

sure that you stay organized so that samples are plated on the properly labeled petri dishes.

17.6.3 Spread plate .1 mL of the vortexed spore suspension.

17.7 CDC Stomacher Method for Processing for Wipe samples

17.7.1 Purpose

This procedure serves to recover and isolate spore forming bacteria from environmental smooth non-porous surfaces and personnel hands. The procedure is used to assist in defining the role of environmental contamination in transmission of healthcare associated infections.

17.7.2 Reagents

Sterile phosphate buffered saline with 0.02% Tween 80 (PBST; Product # 0082, CDC Scientific Resources Program) or equivalent
Appropriate media for optimum recovery of suspected organism. Example:

- Bacterial recovery (facultative anaerobe): BBL™ Trypticase™ Soy Agar with 5% Sheep Blood (BAP; BD) or equivalent
- Butterfield phosphate buffer (BB) 9 mL tubes (sterile; Becton Dickinson (BD), Sparks MD)
- Sterile water

17.7.3 Equipment

- Centrifuge
- Vortexer
- Stomacher

17.7.4 Supplies

- Sterile Stomacher bags
- Sterile pipettes: 50 ml and 5 ml Pipettors (1000 and 200-µl maximum volume) and sterile tips
- Hockey-spreaders (sterile)
- Sterile reusable forceps

17.7.5 Specimen

- Surface wipe samples: cellulose sponge, gauze or equivalent

17.7.6 Procedure: Stomacher Processing

- With sterile forceps, remove sampling wipe and place in stomacher bag accordingly. Do not discard forceps since it will be used later, store in wrapping

- Add 100 ml of PBDW to each stomacher bag with sterile 50-ml pipettor
- Place stomacher bag in stomacher and homogenize for 1 min. at 260 rpm
- Remove stomacher bag and squeeze excess fluid from wipe. With forceps used in Step 3, remove wipe and place back in original sample container. Place reusable forceps in discard.
- Allow foam to reduce in homogenate for about 5 minutes.
- From "Untreated" sample, remove 0.1 ml from undiluted suspension and inoculate on selected media (10^{-1} dilution); and remove 0.1 ml from 1:10 dilution and inoculate on selected media (10^{-2} dilution). Perform in triplicate. Spread with sterile hockey sticks.

17.7.7 Procedure: B - Recovery (Quantitative and Qualitative) – Facultative Anaerobe/Aerobes

- After incubation period, remove media and examine for growth.
- Observe and count colony forming units (cfu) and record on agar plate and recording form
- Observe and count colony forming units (cfu) and record on agar plate and recording form

17.8 Interpretation/Results

- Determine # CFU /Sponge or square area sampled from plates with 25 – 250 colonies:
- Report the final total #cfu recovered/wipe or area sampled
- If no growth was as less than the detection limit (10 cfu).

18.0 Quality Control and Quality Assurance

QA/QC samples and procedures listed in section 6.0 will be followed.

19.0 Data Analysis

Data analysis procedures discussed in section 3 of the QAPP will be used.

20.0 Water Parameter Monitoring

Water parameters outlined in section 5.0 will be monitored for all tap water used in the study.

Appendix C: PREPARATION OF REAGENT WATER AND MEDIA

5.0 Scope and Application

The procedures in this appendix specify how reagent water and reagents will be prepared. Reagent-grade chemicals shall be used in all tests. Included in this appendix are instructions on microbiological media preparation and instructions on how water with various ionic strength and pH values will be made. Lastly, in order to expedite biofilm growth, acetate will be added to tap water that is in contact with the coupons.

6.0 Health and Safety Warnings

- Observe all safety procedures discussed in the QAPP HASP in addition to any site-specific safety considerations.
- Disinfect laboratory equipment and benches daily.
- Report all accidents to the ORD safety manager.

MSDS sheets detailing the hazards of media and reagents are available in the laboratory

7.0 Reagent-grade Water

Reagent-grade water will conform to specifications in Standard Methods for the Examination of Water and Wastewater (21st ed.).

7.1 Phosphate buffered dilution water (PBDW)

7.1.1 Stock potassium phosphate solution

Potassium phosphate (KH_2PO_4)	0.34 g
Reagent-grade water	~1.0 L

7.1.2 Procedure:

- Combine potassium phosphate and 500 ml of deionized water and stir to dissolve in a 2 L bottle.
- Adjust pH to 7.2±0.5 with 19 N NaOH
- Bring volume to 1L with deionized water. Stir to combine.
- Autoclave for 20 minutes at 121º C.
- Store at 4ºC.

7.1.3 Stock magnesium chloride solution

Magnesium chloride hexahydrate (MgCl2 6 H2O) 81.1 g
Deionized water

- Combine and stir until dissolved in a 2L bottle.
- Autoclave for 20 minutes at 121ºC.
- Store at 4ºC.

7.1.4 Working PBDW solution

Stock potassium phosphate solution
1.25 ml
Stock magnesium chloride solution
5.0ml
Deionized water
1L

7.1.5 Procedure:

- Combine ingredients and stir to mix
- Autoclave at 121º C for 30 min
- Store at room temperature.

7.2 Extraction Buffer With Tween

7.2.1 Composition

PBDW	~1.0 L
Tween 80	0.1 mL (0.01%)

7.2.2 Procedure:

- Fill 2-L bottle with 500 mL of PBDW.
- Add pipet 100 ul of Tween 80.
- Bring volume to 1 L with reagent-grade water. Stir to combine.

8.0 Spore Preparation Procedure

8.1 Slants of *Bacillus* on Heart Infusion (BHIA) agar are stored in the refrigerator. To prepare new spores, a fresh transfer of *B. anthracis* is streaked into a slant of BHIA and incubated overnight at 25ºC. This fresh culture is used to inoculate the spore media.

8.2 Growth of Spores

8.2.1 Inoculate 500 mL Erlenmeyer flasks containing 100 mL Generic Spore Media (see formulation below) with a culture of vegetable cells of *Bacillus.*

8.2.2 Incubate with continuous, gentle shaking at 35º for at least five days.

8.2.3 Check the solution for the presence of spores with a wet mount slide preparation using phase contrast microscopy.

8.2.4 When the slide preparation reveals an adequate spore suspension, proceed with the purification.

8.3 Purification of Spores

8.3.1 Ascetically transfer the contents of each flask into sterile 25-mL centrifuge tubes. Balance the tubes and centrifuge at approximately 5900 fcf for 20 minutes, using a fixed-angle rotor.

8.3.2 Pour off the supernatant into a discard beaker. Add 30 mL of cold, sterile deionizer water to each tube. Vortex each tube until the pellet is completely resuspended in the water. Centrifuge again at approximately 5900 rcf for 10 minutes. Discard the supernatant and resuspended in 30 mL of cold, sterile deionized water per tube. Centrifuge for another 10 minutes as before, and discard the supernatant.

8.3.3 Combine the contents of the tubes into multiples that will allow ease of centrifugation. Aseptically add 30 ML of cold, sterile deionized water to each tube and resuspend the spores.

8.3.4 Combine 58 mL of Hypaque™ with 42 mL of sterile, deionized water. Mix well. Add 12 mL of the Hypaque™ solution to a clean, sterile, 35-mL centrifuge tubes. Pipette the spore suspension, carefully layering it on top of the Hypaque™ solution. Centrifuge at approximately 5900 rcf for 30 minutes, using a swinging bucket rotor.

8.3.5 Pour off and discard the supernatant. Add 30 mL of cold, sterile deionized water to the pellet in each tube and resuspend the spores. Centrifuge at 5900 rcf for 15 minutes, using a fixed-angle rotor.

8.3.6 Discard the supernatant and resuspend the spores in 30 mL of cold, sterile deionized water. Wash the spores by centrifuging at 5900 rcf and resuspending twice more. Centrifuge again, discard the supernatant, and resuspend the spores in a 40% (v/v) ethanol solution. Store at 4ºC.

9.0 Waste Management

Dispose of all samples, solvents, reagents and laboratory wastes in accordance with the Laboratory's Waste Management Guidelines.