THE USE OF SPIDER WEBS AS PASSIVE BIOAEROSOL COLLECTORS

THESIS

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Abstract

The threat of attacks using chemical, biological, radiological and nuclear (CBRN) material continues to garner widespread international attention. Despite advances in CBRN protection technologies, many areas remain vulnerable. Bioterrorism, particularly, is an area of concern as potential genetically engineered pathogens, coupled with recognized biological warfare agents, could cause economic, physical, and psychological distress. In the future, inexpensive natural passive collection methodologies may find application in complementing state of the art technologies, establishing contamination boundaries, and providing post incident historical data. The purpose of this research was to determine if spider webs could be used as natural passive bioaerosol collectors. Spider webs were suitable collectors of aerosolized microorganisms in different locations and under different environmental conditions. The webs collected both bacteria and fungi. Microbial growth recovered from the silk fibrils seemed to reflect background species. Multiple environmental factors impacted this study; however, solar radiation was considered the most influential factor. In areas where solar radiation was assumed highest, the number colony density decreased; however, the variations were not considered statistically different. Spider webs' ubiquitous nature makes them a suitable proxy, not only in the detection of possible pathogens, but possibly in the collection of chemical and radioactive fallout.

To my wife and children

Acknowledgments

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Table of Content

Page

Page

Page

List of Figures

Figure Page

Figure Page

List of Tables

THE USE OF SPIDER WEBS AS PASSIVE BIOAEROSOL COLLECTORS

I. Introduction:

1.1 Background

Biological weapons have been a concern of our leaders for decades. Shortly after his inauguration, President Nixon declared that the United States unilaterally renounced first use of lethal or incapacitating chemical agents and unconditionally renounced all methods of biological warfare (BW). After the announcement, the U.S. involvement with biological weapons was confined to research on strictly defined measures of defense, such as immunization. In 1972, the U.S. and the Union of Soviet Socialist Republics (USSR) agreed in principle to a document banning the development and use of biological weapons. Under the terms of the agreement, the parties agreed not to develop, produce, stockpile, or acquire biological agents or toxins of types and in quantities that have no justification for prophylactic and other peaceful purposes [49].

The USSR exponentially expanded their offensive bioweapons program under the cloak of this treaty [1]. In his book *Biohazard*, Russian defector and senior scientist Ken Alibek provides chilling details of the USSR's advanced biological weaponry. Many pathogens thought too unstable to weaponize were weaponized and sophisticated delivery platforms placed every corner of the world within striking distance [1]. During the emergence of Russia in the 1990s, it is suspected that many BW weapon secrets were sold to rogue states by out of work and disgruntled Russian scientists. Other weapons

may have been secretly transferred to secure locations within Europe, Asia, and the Middle East to avoid scrutiny and accountability. The final destination or disposition of this massive weapon stockpile remains a mystery.

On September 11, 2001, attacks on the World Trade Center in New York City, The Pentagon in Washington D.C., and Somerset County Pennsylvania launched the U.S. into a new asymmetrical global war on terrorism (GWT). The level of sophistication demonstrated by Al Qaeda during the attacks and their devastating consequences highlighted several vulnerabilities. First, foreign terrorists had infiltrated the U.S. and for years had observed, trained, and planned some of their actions from within our borders. Second, intelligence gathering and information management systems had deteriorated over the years. Lastly, detection techniques for CBRN threats were not adequate to protect the nation from future threats. The lack of accountability of soviet BW agents, the U.S. evident vulnerabilities to CBRN threats and the advent of global terrorism make the development of inexpensive and innovative BW detection techniques absolutely necessary.

1.2 Research Objective

This project addressed the feasibility of inexpensive passive collection techniques for augmenting state of the art monitoring initiatives, the possible use of spider webs as passive collectors of bioaerosols regardless of location, e.g. rural, urban, or forested. The ultimate objective was to determine if microbes, regardless of species, could be recovered from spider webs. Pathogenic species described in the Centers for Disease Control and Prevention (CDC) select agent list, a list of 38 biological agents and toxins that have the

potential to pose a severe threat to public health and safety [50,55], are the eventual target population of this project. However, this research was limited to microorganisms present in bioaerosols in areas near Wright-Patterson Air Force Base, not select agents or any of their surrogates. Positive identification and classification of the observed species was beyond the scope of this initial study.

1.3 Motivation

Interest in passive detection methods originated from shortcomings in the BioWatch program. In July, 2008 the Government Accountability Office (GAO) reported that, although significant advances had been made in the Department of Homeland Defense (DHS) next generation of BW detectors, they may not be ready for initial deployment until 2010 [21]. Operational and manufacturing costs may restrict the initial acquisition of enhanced detectors to 100 [21]. This limited number of detectors may not be sufficient to effectively protect even one large U.S. city. Spider webs may serve as additional sample collection mechanisms in areas not covered under the DHS BioWatch program, to help determine and define affected boundaries in the event of an attack, or, to provide confirmatory data after a release is detected in protected areas. The spider webs' ubiquitous nature, strong collection properties, and rather inexpensive analytical requirements make them a practical proxy for BioWatch sensors.

1.4 Previous Work:

It appears that the objective of this experiment has not been explored before, that is, the use spider silks to collect airborne microorganisms. In experiments conducted in New South Wales, Australia, spider webs were used to determine, via atomic absorption

spectroscopy and ion chromatography, the lead and zinc pollution created by vehicular traffic [20]. Researchers concluded that the collection capacity of the spider webs did not degrade with time, i.e. old silks continued to collect ions, and the collection capabilities of silks were comparable to other methodologies [20]. In Lucknow, China, researchers used spider webs to determine regional flora by analyzing pollen deposition on spider webs [2]. Researchers in this study statistically compared the pollen concentration recovered from silks with results obtained using other volumetric techniques and found that there was not a statistical difference between the methods [2]. Consultation with multiple subject matter experts on spiders and spider webs revealed no additional work using spider webs as passive natural collectors.

1.5 Methodology

The objective was binary in nature; can microorganisms attach to spider webs, and, can they be recovered? In the previous experiments where spider webs were used as collection mechanisms, the targeted population was abiotic. In this study, the destructive analytical methods from the previous studies could not be used as our target populations, microorganisms, are susceptible to chemical and heat treatments. Microbial culture offered a solid scientific approach to satisfy the research objective of determining if microorganisms attached to spider webs. Culture techniques, proven versatility in the study of a wide variety of microorganisms, made them an obvious choice for this initial study. Two general agar-based growth mediums; Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA), each addressing microorganisms of interest, were used. We used microscopy cover glass slides as the silk collection apparatus. The webs' surface tension

allowed undisturbed attachment to the cover glass slide and immediate deposition onto one of the growth mediums. After growth was visible, Gram stains, an empirical method of differentiating bacterial species based on the chemical and physical properties of the cell walls, were used to classify the populations. Limited statistical comparison and analysis was done to determine the possible impact of seasonal variations in webs collection properties and a possible saturation point.

1.6 Assumptions and Limitations

Sample collection was done under normal environmental conditions in four locations near Wright-Patterson Air Base, OH. A collection scenario was used similar to that in which a first responder would attempt to collect spider webs for analysis after an attack or during periodic monitoring. The attached particulates and microorganisms were assumed to be unaffected by interactions with the web matrix, as indicated by Hose *et al*. [20]. Bacteria concentrations in air are known to be around 10^6 cells/m^3 with fungi concentration being approximately one order of magnitude less. Background contamination in samples was assumed to be effectively reduced by minimizing media exposure time to ambient conditions. The agar-based growth mediums were not exposed to ambient conditions for more than five seconds during sampling. Despite this small exposure, the level of background contamination or background cross-contamination on the microscopy slide could not be statistically defined. The microscopy slides used for collection offered a uniform counting area where growth could be observed using light microscopy. Any growth observed underneath the slide was attributed to microorganisms present on the web and no background contamination was assumed. Also, oxygen

supply, necessary for aerobic growth, underneath the slide was assumed to be ample throughout the entire observation period. Again, the exact oxygen availability under the slide, or, its uniformity and distribution across the area was not measured. The differences in oxygen availability may have influenced the observed microbial speciation.

1.7 Significance of Results

The findings of this experiment should impact areas of counterterrorism, homeland defense, and possibly forensic investigations. The DHS BioWatch program is costly and is presently deployed only in large metropolitan areas and national high value targets [43]. This research demonstrated that passive natural collectors may be a viable proxy for, or supplement to, expensive detection technologies. Spider webs could be used to randomly sample several areas at a significantly reduced cost. Also, the process may serve to confirm positive results from the more sophisticated BioWatch sensors. Since sensors are deployed in fixed locations, spider webs could be used to determine contaminated boundaries and direction vectors. They may also serve to cover geographical gaps inherent under the BioWatch deployment guidelines. This experiment is a first step in what could be a long examination of the feasibility of utilizing spider webs and other natural media as passive collection mechanisms. Culture techniques revealed a wide range of microbial growth. The use of spider webs as passive collectors can now be expanded by employing more sophisticated techniques to produce reliable quantitative and qualitative results. The approach may also find applications in the detection of chemical agents (CA) and radioactive fallout as well as other intelligence gathering initiatives.

II. Literature Review

2.1 Introduction

The threat of a biological attack has risen with the expansion and power projection of global terrorism. In 2004, the Homeland Security Presidential Directive 10 (HSPD-10) addressed several concerns regarding biological weapons and the danger they represent [3]. The CDC's select agent list focused post 9-11 research objectives on some of the most lethal pathogens that could cause severe damage to people, plants, and animals. The CDC rigorously regulates the use of these select agents through the National Select Agent Registry Program [50,55]. Despite government oversight, accidents have occurred that undermine the regulations and guidelines established by CDC [22]. *Bacillus anthracis*, causative agent for anthrax, has remained a top research priority after letters containing powdered anthrax were delivered to Senators Thomas Daschle (D–SD) and Patrick Leahy (D–VT) in the fall of 2001. The development of additional detection capabilities will enhance the United States' ability to protect its citizens against the evolving BW threat [3].

Two types of detection initiatives must be developed; a clinical and a field detection capability [38]. A clinical detection capability requires alert physicians and proper information exchange to identify biological warfare (BW) event [38]. O'Toole, in her testimony to the U.S. House of Representatives stated that "A covert bioterrorist attack would likely come to attention gradually, as doctors became aware of an accumulation of inexplicable deaths and illnesses among previously healthy people." However, many hospitals are unaware of federal and state reporting procedures when a

BW attack is suspected [38]. Detection and positive identification remain the best alternative to minimize casualties during a BW event. The Science and Technology (S&T) division of DHS has made great advancements in developing the next generation of biological agents detectors; however, several additional vulnerabilities must be addressed to cope with future threats [21]. Gaps in the U.S. monitoring grid, coupled with large unprotected populated areas around the nation, present easy targets to terrorists [43]. Improving existing detection technologies, as well as developing complimentary inexpensive detection and collection methodologies, must be a priority in future initiatives.

2.2 Current Detection Technologies

Biowarfare detection technologies have increased in numbers and evolved significantly over the years. With the increase in recent threats, the need for detection technologies that are accessible, cost effective, and reliable is paramount [21]. Historically, federal agencies independently dealt with the threat of BW. However, new initiatives are driving research and development of newer technologies as a joint effort directed by the DHS. On May 2003, the Department of Health and Human Services (HHS) announced the allocation of $1.4 billion for federally sponsored bioterrorism cooperative agreements [21]. DHS' next generation of BW detectors will have the capacity of collecting, quantifying biomass, in-situ identification, and rapid information dissemination [4]. Legacy technologies could remain in use after the deployment of new detectors and may influence their capabilities [4]. Legacy technologies include the

Biological Aerosol Sentry and Information System (BASIS) and the Biological Integrated Detection System (BIDS) among others.

BASIS is a portable sampling unit, developed by a joint endeavor between Los Alamos National Laboratories and Lawrence Livermore National Laboratories, capable of detecting and identifying airborne biological incidents. Designed for indoor or outdoor use, BASIS was successfully tested with live microbes inside a sealed chamber at the U.S. Army's Dugway Proving Ground in 2001 [4,56]. It has also being successfully deployed during large sporting and political events [4,56]. The system operates by suctioning air into the unit and segregating samples using filters of different sizes which are later analyzed using polymerase chain reaction (PCR) for pathogen DNA signatures. It can accurately estimate exposure levels, critical in facilitating a proper response [4]. The entire process from sample collection to identification typically requires between 8 to 10 hours [56].

In contrast to BASIS; BIDS is a mobile detection platform capable of detecting pathogens in forward locations in support of U.S. Army combat operations. BIDS uses the Joint Biological Point Detection System (JBPDS) as detection mechanism, Fig. 1a, mounted on a M1152 High Mobility Multipurpose Wheeled Vehicle (HMMWV) Fig. 1b [57, 58]. The JBPDS provides detection and identification of airborne biological agents at very low levels, initiates warning systems, and communicates threat information within 15 minutes of detection [58]. When the system detects microorganism of a suspicious nature the collector activates a system that samples hundreds of liters of air per minute. This action significantly increases the number of agents that could be identified by BIDS [58].

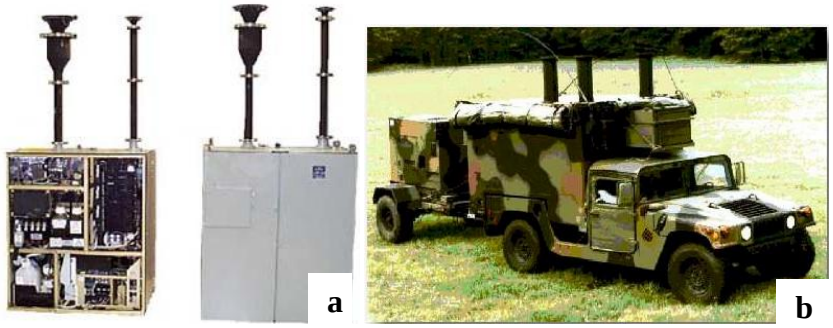

Figure 1. (a) Joint Biological Point Detection; (b) Mobile BIDS unit [57]

The before mentioned legacy technologies contributed components to the DHS' new BW detectors. Future financial incentives may favor the development of newer and better collection and identification instruments; however, the use of legacy technologies like BIDS and BASIS are expected to remain critical for many years.

2.3 Department of Homeland Defense BioWatch Program

The attacks of 9-11 triggered national levels of distress, anxiety, and helplessness not seen since the attack on Pearl Harbor. As part of the federal protection initiatives, the DHS was created and charged with three primary responsibilities; (1) preventing terrorist attacks in the US, (2) reducing vulnerabilities to terrorism, (3) minimizing the damage from potential attacks and natural disasters [4]. Since its creation, the DHS has invested significant funds on research and development projects targeting bioterrorism [31]. The GAO reported in July 2008 that DHS has made significant progress on the establishment of the National Biosurveillance Integration Center (NBIC). This center is intended to streamline the information gathering and sharing among federal agencies regarding biological agents [21]. However, the BioWatch program, which is expected to deploy detectors to collect, analyze, and identify select agents, continues to suffer setbacks [21].

DHS's office of the inspector general (OIG) identified BioWatch's responsibilities as; (1) providing early warning against biological attacks, (2) assist in establishing forensic evidence on the source, nature and extent of biological attacks, and (3) determine the preliminary spatial distribution of biological contamination [44]. Three different federal agencies have key roles in BioWatch operations; (1) sampling- conducted by the Environmental Protection Agency (EPA), (2) analysis- conducted by CDC designated laboratories, and (3) response- conducted by the Federal Bureau of Investigation (FBI) [43]. Shea and Lister's document highlighted several controversial points within the program. First, the program's current operational area is predominantly large cities and high value targets, e.g. national labs. The GAO reported in July 2008, that BioWatch detectors are only deployed in 30 locations across the U.S. [21]. To an extent, this first point neglects protection for smaller cities and rural areas. Due to the secrecy of the program, the selection parameters for coverage are not readily available, creating uncertainty as to the surveillance criteria. Second, since EPA is in charge of sample collection, the EPA collocated the BioWatch detectors with the agency's pollution monitors [32,43]. The EPA interest, pollution, may not correlate with the interest of DHS, bioterrorism. Third, there are gaps within the monitoring area. Each sensor's operational cost is approximately $41,000 per year with an acquisition cost of approximately $90,000 per unit [21]. The high cost associated with the detectors may restrict the initial acquisition to 100 enhanced detectors [21]. This limited number may not be sufficient to protect a large city and will leave the majority of the nation vulnerable to BW attacks.

2.3.1 Description of BioWatch Sensor Network.

The HSPD-10 emphasized the BioWatch sensor network as one of the greatest initiatives of the federal government against bioterrorism [3]. Current BioWatch initiatives feature elements of the BASIS and BIDS technologies while using laboratories, part of the federal laboratory response network (LRN), for genetic analysis and identification. The detectors collect bioaerosol samples through a sophisticated set of filters that rotate every hour [43]. Filter retrieval occurs every 24 hours creating concerns pertaining chain of custody and possible cross-contamination during transportation [43,44]. LRN laboratories use polymerase chain reaction (PCR) to positively identify pathogens; however, toxins and viral pathogens may be beyond the detectors limit of detection [6]. The analysis process takes between 10 to 34 hours [21]. On Feb 12, 2002, a sample collected at an airport near Salt Lake City, Utah indicated a positive result on more than one single-strand DNA test [40]. Upon notification, airport officials decided to wait for confirmatory tests before implementing a costly response plan [40]. The confirmatory test, which is expected to be a requirement in future scenarios, revealed a cross-reacting non-pathogenic organism [40]. Questions regarding the reliability of BioWatch detectors ensued following this false positive. DHS' next generation of detectors will incorporate the Autonomous Pathogen Detection System (APDS) developed by Lawrence Livermore National Laboratory (LLNL) [6]. APDS uses immunoassays and PCR analysis to conduct in situ tests on bioaerosols samples without human involvement, therefore removing chain of custody concerns [6,19]. These detectors are expected to be fully deployed by 2013 [21]. Cost will continue to be the limiting factor for the effective deployment of BioWatch sensors. JASON, a scientific

advisory group, estimates that effective nationwide coverage would cost $10–15 billion, which would be a significant expansion over BioWatch's current $85 million annual budget [21]. Despite extensive monetary investments and the use of the latest technologies false positives still may continue to occur, coverage gaps may be extensive, and sensor emplacement may be inadequate. The need for inexpensive detection and collection technologies to provide protection for rural areas, as well as confirmatory tests in protected areas, may find answers in unorthodox collection mechanisms.

2.4 Spider and Spider Webs Characteristics

The extraordinary properties of spider silks have marveled engineers and scientist for years. Spider silks, despite their slender surface and size, have tensile strength akin to some of the strongest material developed in modern laboratories, Table 1.

Table 1. Comparison of Mechanical Properties of Spider Silks [26]

Material	Strength ($N\ m^{-2}$)	Elongation (%)	Breaking Energy ($J\ Kg^{-1}$)
Dragline silk	4.0×10^9	35	4.0×10^5
Minor Ampullate Silk	1.0×10^9	5	3.0×10^4
Flagelliform Silk	1.0×10^9	> 200	4.0×10^5
Tubuliform	1.0×10^9	20	1.0×10^5
Acinoform	0.7×10^9	80	6.0×10^9
Kevlar	4.0×10^9	5	3.0×10^4
Rubber	1.0×10^6	600	8.0×10^4
Tendon	1.0×10^6	5	5.0×10^3

The interest in spider silks has been to improve structural construction designs and to develop lighter and stronger fibers. With recent advances in technology, scientists were able to characterize the chemical composition of numerous spider silks. The major

constituents of silks are proteins, which are synthesized in specialized glands and are of high molecular weight.

There are two main categories of spider webs, cribellar and escribellar (viscous capture threads) [41]. The cribellar spiders possibly form the oldest species of arachnids currently in existence [15]. These spiders have an organ known as the cribellum, Fig. 2 that functions as a comb [48].

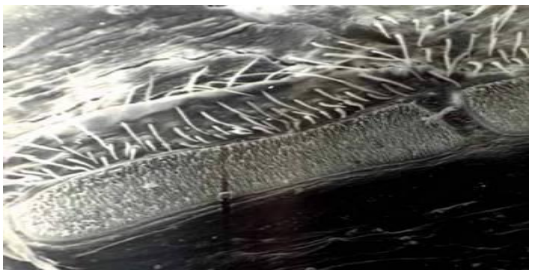

Figure 2. Cribellum Organ [59]

As the spider passes the fibrils through the cribellum the combing process also imparts electric charges to the thread which makes the puffs repel each other while maintaining structural integrity [48]. These fibrils later serve as a trap that can easily entangle small prey and particulates without the need of adhesive properties [36]. Thread capturing capability is determined by the number of fibrils that form the thread and is modified by the dimensions of puffs and the manner in which the spider geometrically manufactures the thread [15]. There are two types of cribellate webs; noded and non-noded. These webs have distinctive adhesive properties dictated by the presence or absence of the nodes [15]. Hawthorn and Opell demonstrated that the adhesive properties of non-noded, Fig. 3(b) web remains constant during different periods of relative humidity as its adhesive properties are dictated by Van der Waals forces [15]. Noded silks, Fig. 3(c)

employ hygroscopic forces as their adhesive mechanism and are significantly influenced by relative humidity [15].

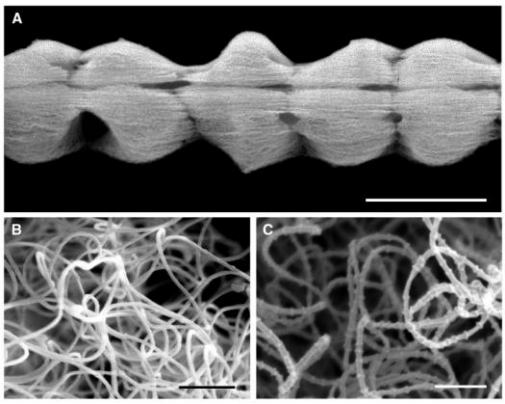

Figure 3. (a) Cribellar thread of *Hyptiotes cavatus*; (b) Cylindrical Cribellar Fibrils of *Hypochilus Pococki*;(c) Noded Fibrils of *Hypotiotes Cavatus* [15]

At low relative humidity both, type of silks have comparable adhesive properties with Van der Waals forces dominating the adhesive mechanism [15]. However, as the relative humidity increases nodded cribellate webs have stronger adhesive properties due to an increase in hygroscopic forces [15].

The escribellar orb web is spun from two different glands, flagelliform and aggregate glands, in the escribellate spider. As the spider weaves the web the aggregate glands coat the fibers with a complex aqueous solution, composed of glycoprotein, that later develop into hydrophylic droplets which exhibit strong adhesive properties [35]. The adhesive properties of viscous capture threads, Fig. 4, are superior to that of cribellar, noded and non-noded. Viscous capture threads on average achieve 13 times more adhesion than cribellar threads [35]. The wet viscous capture thread of the ecribellate orb can also extend several times its original length while dry cribellate silks are far less elastic [48]. The protein composition of spider webs is affected by the animal's diet with

extensive variations possible [9]. The process of creating a new web consumes many critical compounds essential for the spider's survival [18].

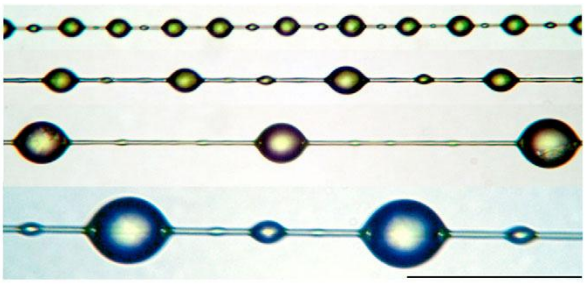

Figure 4. Viscous Captured Threads [35]

To replenish lost metabolic nutrients during silk manufacturing, spiders consumed their old silks and captured prey daily [9,18]. Craig *et al*., in an experiment in 2000, showed how extensive silk protein composition varies with changes in amount and insect species fed to spiders during a controlled experiment [9]. Vollrath *et al*., in a separate experiment, showed that nutrient depletion could affect silk mechanical properties [9]. There is also evidence that spiders may alter their web's composition based on their physical surroundings requirements [9,18]. A silk's amino acid composition may be use to determine insect populations in an area as well as possible silk mechanical properties [9]. Higgins *et al*., noted that dietary and environmental deviations affect the webs adhesive properties and consequently its capturing capability [18]. Webs should collect microorganisms present in bioaerosols despite variations in silk composition as a consequence of the small size and high concentrations of microorganisms.

2.5 Bioaerosol Dispersion

Microbial airborne movement has gained wide spread attention with the occurrence of global pandemics and the advent of global terrorism. The atmosphere is

filled with a myriad of bacteria and fungi species able to survive despite extreme temperatures, intense solar radiation, and a nutrient depleted environment; all capable of inactivating cells [13]. Airborne cells are also susceptible to other environmental factors such as relative humidity (RH), temperature, and pollution. The aerial dispersion of microorganisms causes annual losses, ranging in the billions of dollars, in agricultural and livestock industries [27]. Spores particularly are highly resistant forms of microorganism that are capable of remaining in the atmosphere for an extended period of time [2,7]. The speciation of bioaerosols is highly variable and it is influenced by type of microorganism, particle composition of air, and environmental gasses present in the air [28]. Seasonal temperatures influence the viability of bioaerosols with Gram-negative bacteria prevalent during warmer temperatures [47]. The size of bioaerosols ranges from 1 μm to 100 μm and microorganisms can exist as stand-alone entities or could be present in suspended abiotic particulates [29].

The boundary layer, located approximately 0.1 kilometers above a surface, probably contains the highest population of airborne microbes and is responsible for the majority of microbial dispersion [29]. Recently, scientists coined the term long-distance dispersal (LDD) to describe microbial dispersion. There are two types of microbial dispersion patterns that command attention; (1) single step movement, airborne microorganism move to one area, settle, and replicate, and (2) range expansions, the slow gradual atmospheric movement through a region [7]. Single step movement is extremely rare and difficult to predict as its dynamics require abnormal meteorological events akin to "el Niño." Microbial range expansion is relatively predictable and can spread though air, water, ground, or commercial shipments [7]. The movement of bioaerosols is

affected by multiple forces such as diffusion, inactivation, and deposition rates [29]. Bioaerosols diffusion is strongly correlated to airflow and atmospheric turbulence [29]. Without atmospheric turbulence, bioaerosols follow a constant stream downwind, not Brownian motion [29]. Physical structures also impact diffusion by altering airflows and, consequently, movement vectors. Deposition occurs via multiple factors such as, gravitational settling, downward diffusion, rain deposition, and surface impaction, among others [29]. Modeling accurate microbial dispersion faces extensive limitations despite technological advancement. The high variability in speciation and the microorganisms' proven susceptibility to environmental factors create a highly complex system to model [24]. The complicated movement patterns, inactivation factors, and variable deposition rates make bioaerosols a highly unpredictable field of study.

2.6 Microbial Sources and Environmental Factors Interactions

Microbial communities in urban and rural bioaerosols have an amalgam of unknown source terms. Linear sources and point sources are the principle contributors of microorganisms to bioaerosols [29]. Linear sources follow wave function dispersion patterns whereas point sources follow conical dispersion patterns [29]. The process by which particulate and microbes are aerosolized is called launching [29]. Aerosolized microbes seldom result in severe health effects [47]. Water reclamation and soil tilling are some of the sources that are known to launch extensive amounts of microbes into the air [34]. Diffusion and atmospheric turbulence determined the distance travelled by microorganisms and their ultimate suspended time while environmental factors particularly RH, wind velocity (WV), and solar radiation (SR) influence cell viability and

concentration [24]. WV has the most pronounced effect on bacteria concentration as it impacts bacteria decay rates and may determine deposition rates [34]. The latest computer models suffer from large biases due to the inability to properly identify source strength terms and the inability of predicting environmental effects on cell viability [42]. Also, air pollutants present in the atmosphere may have a direct effect on bacteria viability [30]. The aforementioned complexities make bioaerosols an interesting field of study with direct impact on human health, economic development and national security.

2.6.1 Bioaerosol Composition near Waste Water Treatment Facilities

The population expansion of the past 50 years has made water reclamation a necessity. The objective of water reclamation is to remove contaminants present in wastewaters by chemically and biologically treating the water until the product is a combination of clean water, released to water effluents, and sludge used in agriculture as fertilizer. Due to political and economic constraints, Waste Water Treatment Facilities (WWTF) are often located in close proximity to densely populated areas [11]. The reclamation process is known to aerosolize bacteria by the bursting of air bubbles created through the aeration process [11,42]. When WWTF operations aerosolize microorganisms, the distanced travelled by these microorganisms is normally restricted to approximately < 250 m downwind from the WWTF aeration tanks [11]. Previous studies have proven that there is a significant deviation in the number of enteric bacteria, bacteria originating in the intestines of animals and people, colony forming units in areas upwind and downwind from aeration tanks [42]. Nighttime seems to be a rich environment in

speciation and concentration of microorganisms in areas near a WWTF, with levels significantly surpassing those of background amounts [11].

Table 2. Microbial Counts Observed in Samples from the John E. Egan WWTF [42]

	Counts/m^3 of air			
Run no.	SPC	TC	FC	FS
1	970	28	7	14
2	2,002	298	130	42
3	1,911	346	63	21
4	1,588	133	10	0
5	1,666	42	21	24
6	2,068	410	112	24

Some of the most common aerosolized bacteria resulting from the aeration process include standard plate count (SPC) organisms, total coliforms (TC), fecal coliforms (FC), and fecal streptococci (FS), Table 2, [42]. The amount of aerosolized bacteria created by the aeration process may vary from facility to facility as a result of divergent aeration rates and holding tank sizes [42].

During a study performed in Chicago, Sawyer *et al*., determined the concentration of the before mentioned microorganisms released by a WWTF during organic waste processing operations [42]. The FS and FC reduced concentration were attributed to a higher decay rate for these organisms [42]. Fannin *et. al.*, in a similar experiment, compared the difference in concentrations of aerosolized enteric bacteria near a WWTF during night and day hours prior to the start of operations of a WWTF in the Chicago area, Table 3 [11]. Their results clearly indicate the impact of WWTF operation in aerosols speciation and it also provides insight as to distances travelled by some of these microorganisms. Areas downwind from the processing tanks are expected to yield higher

microorganism concentrations than areas upwind [11]. However, areas in the immediate vicinity of aeration tanks should yield a higher microbial concentration and speciation than areas downwind from aeration tanks [11].

Table 3. Aerosols Density of Bacteria near O'Hare Water Reclamation Plant [11]

	Density (CFU/m^3)							
			Downwind (m)					
Microorganisms	Upwind		< 150		150-250		> 250	
	Day	Night	Day	Night	Day	Night	Day	Night
TC								
Preoperation	0.21	0.28	0.24	0.27	0.28	0.18	0.22	0.12
Postoperation	0.22	0.09	6.81	5.17	0.86	0.57	0.4	0.34
FC								
Preoperation	< 0.04	< 0.06	< 0.03	< .06	< 0.04	< 0.06	< 0.03	< 0.06
Postoperation	0.01	0.01	1.67	2.09	0.18	0.64	0.29	0.15
FS								
Preoperation	0.13	0.88	0.04	0.7	0.14	1	0.06	0.58
Postoperation	0.04	0.83	0.29	2.07	0.15	1.21	0.48	0.86

In night hours, the concentration of bacteria in aerosols increases at most locations within or in the vicinity of a WWTF, table 3, [11]. The increase in bacteria concentrations during night hours could be the result of a decrease in the decay rate or greater atmospheric stability [11].

However, LDD and other human activities, such as agriculture and recreational activities, may also significantly contribute to the final speciation of bacteria in areas near a WWTF [11]. Although, the true concentration of aerosolized bacteria near a WWTF may never be determined, due to the complexity of the system, it is undisputable that the aeration process is a significant source of aerosolized bacteria.

2.6.2 Microbial Dispersion and Viability in Rural Settings

The soil tilling process, breeding, and grazing of farm animals in rural settings create a highly variable bioaerosols environment. When farming and breeding activities change, the amount of microorganisms present in bioaerosols is proportional to the change [27]. Storage of grains and other agricultural products also increases the microbial composition of air samples [27]. For example, the storage of hay is known to be a significant source of Gram-negative bacteria, fungi, and other metabolites [27]. Fungi particularly are of interest in rural environments due to the recorded high levels present in air samples [27]. Little is known as to the true genera or speciation of microbes present in rural areas [27]. It is believed that microorganisms may be susceptible to chemicals used in agriculture as many may be capable of deactivating their replication mechanisms [30]. The impact of chemical fertilizers, insecticides, and fungicides on the ability of bacteria to aerosolize is debatable. Although the true kill rate of chemicals used in agrarian activities is not clearly defined, it is plausible that, given the large amount of bacteria present in soil, aerosolization of microorganisms after chemical applications should continue to be significant in rural settings.

2.6.3 Microbial Dispersion and Viability in Urban Settings

Diversity and concentration of microbial urban air samples is limited, in contrast with rural areas. The limited number of microbial sources decreases the speciation and concentration of microorganisms in urban air samples; however, concentrations can still reach 10^6 cfu/m^3 orders of magnitude [5,30]. Microorganisms in urban settings could have a negative correlation with several airborne pollutants; however, these interactions

remain relatively unknown [30]. Studies conducted by Lighthart and colleagues indicate that aerosolized microorganisms may be susceptible to the common atmospheric pollutants carbon monoxide (CO) and sulfur dioxide (SO_2) [46]. Airborne particulates may increase cell viability by providing shielding from solar radiation [5,30].

Comparison between replicate observations and between experiments is difficult in urban settings due to the high variability of environmental factors in outdoor experiments, difference in speciation in short time intervals, and subjective research parameters employed in controlled laboratory experiments [30]. Studies indicate that Gram positive cocci far outnumbered Gram negative bacteria of any kind in urban bioaerosols [30]. Many significant questions pertaining to microbial speciation, viability, and dispersion remain unanswered. The complexity of the study environment may require that future research utilize nucleic acid technologies for microbial identification and advanced meteorological monitoring stations to study weather effects on dispersion and cell viability.

2.7 Miscellaneous Environmental Effects on Bioaerosols

In previous sections, several environmental factors were linked to cell viability and dispersion. When multiple factors are considered simultaneously, the resulting interactions become extremely complex. Wind velocity, solar radiation, and precipitation are among some of the unpredictable factors that unquestionably influence microbial movement, viability, and dispersion [24]. Despite the stress caused by changing environmental factors, aerosols continue to be densely populated by viable microbes [34]. Weather conditions could largely affect deposition patterns of microorganisms as well as

their dispersion. For example, precipitation may accelerate deposition as bioaerosol particulate may merge with water droplets and fall to the ground at a faster rate; therefore, restricting its dispersion area [29]. Electrostatics also play an important role in bioaerosol deposition. At neutral pH, most bioaerosols tend to have negative surface charge [29]. Interactions with positively charged particulates, caused by shifts in environmental conditions, may cause coagulation and increase deposition rates of bioaerosols [29]. Dry weather conditions may increase microbial dispersion or deactivate sensitive bacteria through desiccation [29]. Relative humidity affects Gram positive and negative bacteria in opposite ways. Gram negative bacteria are deactivated during periods of high RH whereas Gram positive bacteria thrive under these conditions [29]. RH humidity also increases particle size distribution as bacteria tend to coalesce with increases in RH [24].

Solar radiation (SR) is of particular interest when studying airborne microbes. The SR effects are dependent on exposed species, duration of exposure, and radiation intensity [46]. The time of the day is directly correlated with cell viability, as demonstrated by Federak and Westlake in 1978 [12], and validated by Tong and Lighthart in 1997 [46]. Both studies established that bacterial growth is highest in samples collected from areas where SR was shielded by physical structures or vegetation [46]. Shorter UV wavelengths and ionizing radiation such as X-ray are particularly detrimental to cell viability [29].

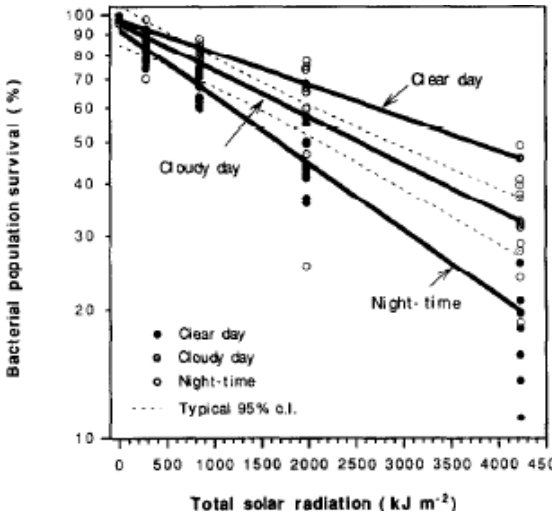

Figure 5. Solar Radiation Effects on Bacterial Population Survival [46]

The incident radiation on viable cells cause DNA damage in the single strand and double strand regions, often resulting in breaks [29]. This inhibits biological activities such as replication, transcription and translation [29]. The lethal effects of SR on bacteria are evident in Fig. 5. It is also evident that bacteria concentration is fairly constant at different times of the day, Table 4.

Table 4. Percent Survival of Outdoor Atmospheric Bacteria Exposed to Summer Noontime Solar Radiation [46]

Exposure time (Min)	SR irradiance 250-1100 nm, kJ m^2	Clear noontime airborne bacteria (n = 8)	Cloudy noontime airborne bacteria (n = 5)	Midnight airborne bacteria (n = 8)
0	0.0	100 ± 0	100 ± 0	100 ± 0
5	281.8	83.9 ± 9.2	86.1 ± 4.0	79.3 ± 4.7
15	845.6	74.8 ± 6.8	79.3 ± 9.5	66.8 ± 5.0
35	1973.0	62.7 ± 16.7	53.4 ± 6.2	43.7 ± 6.4
75	4227.8	32.9 ± 10.4	33.6 ± 4.7	18.1 ± 4.6

mean ± standard error of the mean

Tong and Lighthart's study however, did not identify bacteria genus; it counted colony forming units (CFU) leaving the genus undefined. Fierer *et al*., demonstrated that the phylogenetic variability encountered in the same location is extensive, regardless of time elapsed between samples [13]. Since Tong and Lighthart were unable to establish a phylogentic identification in their study, and Fierer *et al*. described the large variability encountered in samples collected from the same location in 24 hour intervals, it is appropriate to infer that while CFUs remained fairly constant, the microbial speciation changed. This significant fact alludes to possible speciation variability in samples collected from the same site.

2.8 Culture Techniques

Microbial culture is the method normally employed in laboratories to observe and quantify microbial growth under controlled conditions. It finds wide applications in forensics and epidemiological research as growth can be further studied for pathogenic traits [25]. Since its discovery in the later part of the nineteenth century, culture techniques have targeted pure isolates [10]. Culture techniques may underestimate speciation because non-culturable cells could be present in an aerosol sample [47]. Tsai and Macher noted that, in a base study conducted in 100 large cities across the U.S., identification and characterization of cultured bacteria was exceedingly difficult and, despite intense analysis, > 50% of cultured bacteria were unidentifiable [47]. Culture techniques use a predetermined growth medium and incubation temperatures where a targeted microorganism can thrive while other microbial growth is inhibited [10]. Agar is a gelatinous colloidal extraction from red algae used in culture media [10]. Agar's

purpose in a culture study is as a solidifying agent of liquid nutrients that permit the growth of a targeted microbe [10]. With a combination of meat extracts and the addition of chemical agents to a sterilized Petri dish, growth rate and certain behavioral responses of pure microbes can be studied [10]. Years of studies in microbiology have expanded the culture media available for the study of the conceivable infinite number of species present in the environment [10]. Growth media is selected based on the nutrient requirements of the microorganisms of interest [10]. Heterotrophic microorganisms, dependent upon pre-formed organic matter as food source, often require complex nutrients such as peptones and meat extracts while autotrophic microorganisms, capable of growing strictly on inorganic material, often require synthetic media [10]. Several media permit the growth of complex communities. Complex communities highlight microbial diversity in a study; however, it may obfuscate quantitative analysis [10]. Standard Plate Count (SPC) is a regulated quantitative method involving agar based culture techniques [16,17]. The method often requires several dilutions of a pure sample that could be smeared on a media or mixed within the media [16,17]. The inoculated media is then placed in an incubator at a predetermined temperature where growth occurs [17]. The quantification process can executed by automated equipment or by manually counting the colonies formed using a colony counter [16,17]. Surface plate count follows similar procedures to SPC with the exception that inoculation is done by smearing the organisms atop the growth medium. An acceptable number of colonies in a plate is between 15 to 300 colonies per plate [16,17]. Incubation temperatures could be a discriminating factor when using culture techniques as small variation in incubation temperatures may encourage or inhibit growth of species present in a microbial

community [60]. Microorganisms could be grouped by the temperature range in which they grow [60]. For example, psychrophiles are microorganisms that grow at cold temperatures while thermophiles are microorganisms that grow at high temperatures. The range of temperatures microorganism can tolerate is determined by the enzymatic composition of the organisms. Binary cell division, the reproduction mechanism for microorganisms, has an optimum temperature for each species. Culture techniques will continue to find applications in microbiology despite technological advances as their accessibility and reduced cost, coupled with acceptable quantitative results, enables scientist to study the behavior of microorganisms under multiple environmental conditions.

2.9 Summary

The use of spider silks may provide the means for confirmatory analysis after suspected BW attacks. Silks may also serve as passive collection mechanisms that could augment BioWatch detectors in protected areas or they could serve as indigenous collectors in unprotected areas. The shortfalls of the BioWatch program were made clear in multiple articles previously cited. The need to explore inexpensive passive collection techniques is paramount. Whereas BioWatch sensors require the flow of bacteria to pass through its location, spider silks' ubiquitous nature permits collection in a wider area. This performance parameter could be used in establishing contamination boundaries. The survival of aerosolized microorganisms will result from a combination of environmental factors, microorganisms' speciation, and atmospheric chemical composition. False positives may continue to occur in the future despite technological

advances. Due to the high variability in bioaerosols, false positives could only be confirmed with historical data that silks may be able to provide. Financial constraints often require a confirmatory test before a protective response is implemented. Spider silks may be a way of providing an inexpensive confirmatory test while aiding in determining contaminated boundaries.

III. Methodology

3.1 Overview

The primary objective of this experiment was to determine if spider webs could be use as passive bioaerosol collectors. The extensive variability of several environmental factors, as well as changes in the web's protein composition as a result of dietary behavior requires a sequential approach to conclusively determine the silks' suitability as passive collectors. For this study a collection methodology that conserved spider webs, introduced the webs directly onto growth mediums, and permitted in situ light microscopy analysis was developed. No deviations from the developed sampling methodology were necessary at any time. Gold Seal Cover Glass (GSCG) slides, with a surface area of 13.2 cm^2, were used as silk collection apparatus and two generic agar-based culture mediums were used for microbial studies. Webs were collected from four separate sampling areas in urban and rural locations. Each sampling area addressed environmental or physical factors of interest that were unique to each location. Samples were collected between August and September 2008 to minimize seasonal variation bias and microorganisms were allowed to grow at ambient temperatures inside a safety cabinet for a period of five days.

3.2 Growth Medium

The experiment's objective required generic growth mediums in which a large number of aerosolized microbial species could grow without discrimination. Agar-based culture techniques were used to study microbial growth produced by spider webs' fibrils attached to cover glass (CG) slides. Agar is an excellent growth medium because it

dissolves at high temperatures but solidifies at temperatures of less than 45 ^{o}C [10]. Nutrient Agar (NA), Hardy Diagnostics Cat. No: C6461 Lot No: 07115 with an expiration date of Feb 2012, was used as a non selective agent because it permits the growth of a wide variety of nonfastidious bacteria at a pH of 6.8 ± 0.2 [10]. NA consists of peptone, beef extract and agar [53]. The beef extract contains water soluble substances including carbohydrates, vitamins, organic nitrogen compounds and salts. Peptones are the principle sources of organic nitrogen, particularly amino acids and long chained peptides [53]. Sabouraud's Dextrose Agar (SDA), Cole-Parmer Instruments Company Cat No: 14202-62 Lot No: 07116 with an expiration date of Mar 2012, was used for its wide application in mycological analysis [10,54]. Dextrose provide an energy source for the growth of fungi species [10]. SDA is highly selective due to a low pH of 5.6 ± 0.2, which suppresses bacterial growth [10]. The mediums' manufacturers established the agent's mass to deionized water (DI) mixing ratios required to prepare a prescribed number of Petri dishes.

Table 5. Mixing Ratios for the Preparation of 25 Petri Dishes of Growth Medium

	Volume (ml)	Mass (g)
Nutrient Agar	500	32.5
Sabouraud's Dextrose Agar	500	11.5

Each prescribed mass, Table 5, was weighted using a digital balance, Sartorius Element model ELT 602 with 0.01g deviation. The DI volumes were measured using a 500 ml burette, Pyrex® Single Metric Scale, graduated cylinder with 4 ml deviation. The

DI water and agent were poured inside a 1000 ml glass Erlenmeyer flask and manually stirred for about 60 seconds. After mixing, the two Erlenmeyer flasks were sealed with aluminum caps to prevent evaporation and contamination during autoclaving. Once sealed, the flasks were placed atop a stainless steel tray and placed inside an autoclave oven, Tuttnauer Brinkmann model 3870, with a preset autoclaving temperature of 121 ºC and pressure of approximately 15 Bar/ PSI. The autoclaving process lasted for 20 minutes at the preset settings. After autoclaving; the flasks were removed from the oven and allowed to cool at ambient temperature for approximately five minutes. The resulting solution was poured into Petri dishes, Fisher scientific size 100* 15 mm, covered and allowed to solidify for about ten minutes. Each Petri dish was immediately transported to a sampling site or placed inside a low temperature incubator, Fisher Scientific model 146E, at a preset temperature of $4 \pm 0.1\ ^{o}C$ for future use. Growth media were discarded if not used within seven days of preparation.

3.3 Microscopy Slide Sterilization

A sterile collection instrument was required to attribute any observed growth to bioaerosol surface deposition on the spider webs. Previous work involving webs as passive natural collectors dealt with abiotic populations and each study used silk destructive methods [2,20]. In this study, the conservation of the webs was critical and necessary. Gold seal cover glass (GSCG) slides, 22mm wide and 60mm in length, Electron Microscopy Sciences, were used as collection apparatus. The slides' surface area, 13.2 cm^2, permitted in situ microscopy observation without altering the slide position or disturbing microbial growth. Each GSCG slide was immersed in 70% 2-

propanol, General Chemical Corporation, Lot VOC/B/GL, for approximately one minute. The alcohol treatment killed bacteria by disrupting membrane diffusion and by denaturing proteins [8,61]. Some microorganisms such as endospores and mycobacteria are capable of surviving chemical treatments and remain viable [14]. These microorganisms could be deactivated by heat treatments [14]. To assure complete sterilization, cover slides were autoclaved immediately after being immersed in alcohol.

The sterilization process started with CG slides being placed inside 100 ml beakers, that were previously cleaned with soap and tap water, rinsed with DI water, and, lastly, chemically treated with 70% 2-propanol. The CG slides, beakers, and chemically treated aluminum foil were placed on a stainless steel tray and autoclaved. The autoclaving procedure explained in Section 3.2 was also used during slide sterilization. Once the autoclaving process was completed, the beakers containing the CG slides were covered with the chemically treated and autoclaved aluminum foil which served as a shield from the environment. A test involving ten CG slides was conducted to confirm slide sterilization. After completing the sterilization process previously described sealed beakers were moved outside Bldg. 644 for testing. The aluminum foil was broken with sterilized tweezers and one slide was deposited in one of the two growth mediums. None of the test trials showed microbial growth underneath the slide for either medium for a period of 30 days. For transportation, the beakers containing the GSCG slides were placed inside a box, 38.5 cm long x 30.5 cm wide x 25.5cm in height, and secured to prevent damage to the CG slides. The beakers remained closed until a silk was selected for collection.

3.4 Sample Collection

Spiders could be considered nocturnal animals as the night hours bring periods of intense activity. Spiders often consume their silks every 24 hours to replenish expended proteins used during the silk manufacturing process [9,18]. They also rebuild broken fibrils damaged by impact with airborne particulate, consume captured prey, or completely construct new webs during the night [9]. Tong and Lighthart's study indicated that solar radiation decreased the amount of viable bacteria contained in bioaerosols [46]. After several discussions, it was accepted that collection during the morning allowed sufficient time for spiders to complete their rebuilding and dietary requirements and bioaerosols to deposit on the silk before the intense midday solar radiation took effect. Once a silk was selected for collection, two Petri dishes, one Ea NA and SDA, were removed from a Petri dish holding platform, Fig. 6, and placed on top a box along with two beakers containing sterilized CG slides and one Petri dish containing one regular microscopy slide for silk characterization, Fig. 7.

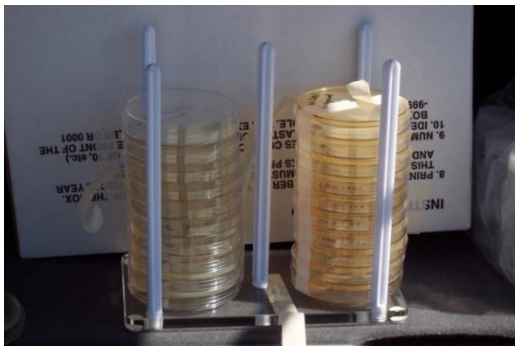

Figure 6. Petri Dish Holding Platform

Each silk sample was collected by swabbing a sterilized CG slide, held by sterilized tweezers, through the silk and immediately depositing the collected fibrils into the agar,

Fig. 7. Five samples for each medium were collected at each location except the parking garage sets in which six samples for each medium were collected on both occasions and the WWTF 500 meter from the aeration basin in which four samples per medium were collected.

Figure 7. Sample Collection at Parking Garage Dayton, Ohio, October 11, 2008

Environmental conditions were monitored during collection using a pocket weather meter, Kestrel® model 4500 NV. After collections, samples were parafilmed in situ and placed in the Petri dish holding platform, Fig. 6, for subsequent transport to the laboratory. Once in the laboratory, all samples were placed inside a safety cabinet, Fisher Hamilton Inc, with a temperature range of 68.69 ± 0.0779 º F.

3.5 Sampling Areas

The spider webs collection capabilities will be influenced by environmental conditions and webs' physical and chemical properties. The region around Wright-Patterson Air base was studied to select areas that reflected urban and rural activities, including agriculture. Four areas were ultimately selected; (1) a forested area, (2) a midsize urban city with moderate traffic volume, (3) a commercial garden center, and finally (4) a waste water treatment facility. These areas provided a range of locations that may be susceptible to agroterrorism and bioterrorism as well as distinctive microbial

launching sources. These areas also had unique physical and environmental characteristics that may impact webs collection capabilities.

3.5.1. Siebenthaler's Garden Center

Siebenthaler's Garden Center is located on Beaver Valley Road in the city of Beavercreek, in Green County Ohio. The garden center has a land area of approximately 450 acres and specializes in shade trees, evergreens, and perennials. This area was selected to study the effects chemicals applied to the plants could have on web's collection capabilities. Traffic volume is significant within the garden center and on adjacent Beaver Valley Rd. The predominant flora in the sampling area was evergreens Fig. 8.

Figure 8. Trail inside Siebenthaler's Garden Center

The webs collected from this site were mainly located on the top of evergreens, Fig. 9 (a) and funnel webs located near the base, Fig. 9 (b).

Figure 9. Garden Center Webs

The center does not use fertilizer or any other chemical substance to maintain inventory production. The contact herbicide Round-up, Monsanto Company, is applied quarterly to inhibit growth of unwanted weeds. Roundup's active ingredient is glyphosate, Fig. 10, which acts by inhibiting enzymatic activity involved in the synthesis of weed's amino acids [51].

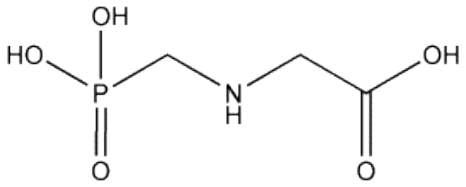

Figure 10. Glyphosate Chemical Structure

Roundup was last applied to the garden center's inventory roughly two months prior to sample collection. The plants are watered at least six times during the summer months. Further watering is not necessary as the garden center is located on top of a large aquifer. No deviations from the methodology described in section 3.3 were necessary. The weather conditions during collection were clear with a sky cover, of 0.0, an average temperature of 70 ^{o}F, and relative humidity of 31 %, [52]. Sky cover is defined as the expected amount of opaque clouds (in percent) covering the sky. Sky cover is reported in tenths, so that 0.0 indicates a clear sky and 1.0 (or 10/10) indicates a completely

covered sky. Sky cover is a factor of concern since it may shield aerosolized microorganisms from the damaging effects of solar radiation. Three background samples were collected by gravity impaction on agar plates. Background samples were collected to obtained insight regarding the bioaerosol composition within the garden center. A similar process was used by Lundholm in 1986 while studying bioaerosols near a WWTF [28]. Petri dishes collecting background samples were exposed to ambient conditions for a period of approximately one hour.

3.5.2. AMPCO Parking Garage, Dayton Ohio

The AMPCO parking garage has eight floors and is located in the middle of the city of Dayton, Ohio, adjacent to the residential building Kettering Tower at the intersection of Jefferson St. and Third Avenue, Fig. 11.

Figure 11. AMPCO Parking Garage, Dayton Ohio

These samples were expected to provide insight regarding webs' collection capabilities despite increases in pollution levels caused by vehicular traffic and exposure to ultraviolet light. The garage experiences high volume usage from Monday to Friday. Usage during the weekend is almost nonexistent. Samples were collected during weekend days at the request of the parking management to avoid interference with

normal business operations. The parking garage was visited on three separate occasions. During the first collection, on August 23, 2008, samples were randomly collected and silks containing high debris content or any discoloration were consciously avoided. The floors used during the first collection were first, second, fourth, fifth, seventh, and eighth. Weather conditions during collection were clear with few visible clouds. The previous 24 hours experienced an average temperature of 78 degrees Fahrenheit, a sky cover of 0.0 and RH of 39% [52]. Three background samples were collected, by gravity impaction, from the second, fifth, and eighth floors.

A second collection was executed on October 11, 2008, following the same floor collection plan used during the first visit. For the second collection, the weather conditions for the previous 24 hours were clear with sky cover 0.1, average temperature of 67 degree Fahrenheit, and an average RH of 50% [52]. The third visit to the parking garage did not involve web collection. A peculiar variability in the number of colonies observed from samples collected at different altitudes motivated a third visit to the garage. To expand on this observation, background samples were collected from all floors on October 24, 2008. The weather conditions for the previous 24 hours were clear with a sky cover of 1.0, average temperature of 49 degrees Fahrenheit, and average RH of 38% [52]. Petri dishes were placed on the north side of the parking garage facing Jefferson St. on top of the garage entrance, Fig 11. Each Petri dish remained exposed to ambient conditions for approximately 1 hour. The samples were collected by gravity impaction. Only the visits in August and early October involved web collection and no deviation from the methodology described in Section 3.3 was necessary.

3.5.3. John Bryan State Park

John Bryan State Park (JBSP) is located in the city of Yellow Springs, in Green County, Ohio. This area was expected to provide insight regarding the effects of SR on web's collection capabilities. Tree canopies were expected to shield cells deposited on the webs from the damaging effects of SR. If true, this natural shield could cause an increase in the number of colonies formed under the slides. The park has a land area of 752 acres and the prevalent outdoor activities are hiking, camping, and biking, Fig. 12.

Figure 12. John Bryan State Park

The tree canopies were dense in the sampling area and debris associated with outdoor activities was visible. The sampling area was located near the main visitor's center in a small depression that contained a small creek in addition to foliage. Samples were collected on August 21, 2008. The weather conditions for the previous 24 hours were clear with a sky cover of 0.0, average temperature of 76 degrees Fahrenheit, and average RH of 38 % [52]. The collected webs appeared recently woven, and webs showing signs of stress, discoloration or high debris content were consciously avoided. This site deviated from the collection methodology in that samples were collected in the

afternoon instead of morning hours to avoid inclement weather conditions expected in upcoming days. Given the shade provided by trees, these deviations were considered negligible. Aside from the time deviation, the methodology described in Section 3.3 was followed.

3.5.4 Green County Waste Water Treatment Plant

The Green County Waste Water Treatment Facility (WWTF) is located on Factory Rd. in the city of Beavercreek, Green County, Ohio. This water reclamation facility processes an average of 8.5 million gallons of water per day (MGD) with a peak processing average of 20.4 MGD in the summer. It has a total of seven basins, Fig. 13, five 93 ft by 24 ft by 15 ft solid waste disposal (SWD) and two 66 ft by 95 ft by 18 ft SWD. The disinfection processes uses 672 ultraviolet light lamps divided into two channels.

Figure 13. Green County Waste Water Treatment Facility Processing Tank

Aeration of this facility is done by six blowers that continuously suction ambient air and pump it into the processing basins. Sample collection was conducted on 15 September 2008, less than 24 hours after the Miami Valley was embattled by remnants of hurricane Ike with recorded wind gusts of up to 75 mph. The previous 24-hours saw

heavy rain, intense winds and power outages that interfered with the facilities regular operations. The average temperature was 75 degrees Fahrenheit. Several areas previously identified as collection sites within the facility were inaccessible due to trees and branches razed by the winds. Most of the silks maintained their full structural integrity despite the high wind velocities. Two areas were ultimately selected for sampling. The first area was close to the aeration basins and the second area was approximately 500 m downwind from the main aeration basins. This sampling location was expected to yield the highest amount of microbial growth since the water reclamation is known to aerosolized a large number of enteric microbes [11,42]. The samples were collected on the same day to minimize possible variability in source term composition as described by Mancinelli [30]. Samples were collected following the methodology described in Section 3.3 without deviations despite persistent winds in the area that in occasions reached gust of up to 15 mph.

3.6 Microbial Growth Quantification

The experiment response variable for this study was average colony forming units per CG slide area. This response variable and developed collection methodology had no precedent in peer-reviewed literature. Accepted microbial quantification methodologies include standard plate counts and flow cytometry among others. Recently, with advances in technology, an enumeration technique using digital camera photos, a chemical dye and an electronic counter is simplifying bacteria enumeration [39]. The quantification methods previously mentioned work best when dealing with pure cultures. Pure cultures were not expected in this study; in fact, a complex growth indicative of the high

speciation variability prevalent in bioaerosols was expected. The enumeration approach minimized disturbance to growing microorganism by avoiding any external contact with the CG slides deposited in the growth medium. Lifting the CG from the media was considered but ultimately rejected as the agar interference, silk debris, coupled with complex microbial communities would yield unreliable counts using the before mentioned quantification methods. The devised enumeration method counted any independent growth formed underneath the CG slides as one colony, regardless of size, and attributed the observed growth to microorganisms attached to the collected webs.

Enumeration was done by using a colony counter, Darkfield Quebec® colony counter model 3325, with a 40 watt standard light source that enhanced the resolution of the counting surface area. Each sample was removed daily from the safety cabinet around 11:00 ± 3 hours for microscopy analysis and colony quantification. The microbial growth was visually inspected and photographed with a digital camera, Kodak easy share DX6440 with four mega pixels with flash setting off. To avoid double counting errors a counting tool using acetate was developed. A cavity with dimensions of one cm wide by four cm long was carved on the acetate paper creating a counting area of four cm^2. This tool was slid through the top of the Petri dish to restrict the counting area and therefore minimizing counting errors. Samples were reintroduced to the safety cabinet each day around 1600 ± 2 hours. The observation process was repeated for a period of five days without deviation. After observation all samples were sealed with all purpose polyvinyl-chloride wrap and placed inside a low temperature incubator at 4 °C.

3.7 Microscopy Analysis

Light microscopy analysis was conducted on every sample throughout the observation period. The analysis was conducted using the Axioskop light microscope, Carl Zeiss Microscope division. Microscopy magnifies images in two steps. First the objective produces a magnified image of the object in the image plane and secondly the eyepiece magnifies the image produced by the objective [23]. Each sample was analyzed in situ by placing the Petri dish atop the microscope stage and adjusting its distance from the objectives and the light source intensity until an adequate visualization of the fibrils and microbial growth was obtained. The Petri dish was manually moved, following the fibrils horizontal contour to obtain counts. Light was projected from the bottom of the microscope into the fibril reaching the CG slide through the agar. The magnifications commonly used were 50 X, 200 X, and 400 X. Identification of the observed growth was beyond the scope of this experiment.

Attempts were made to classify the observed growth as Gram positive or Gram negative to determine if silks discriminated in their collection of biotic microorganisms. Gram staining is an empirical procedure that classifies bacteria into two major groups; Gram positive and Gram negative. Differences in their cell walls causes Gram positive bacteria, which has a thick layer of peptidoglycan in its cell wall, to stain purple or blue, while Gram negative bacteria, which have a thinner content of peptidoglycna to stain red. For this purpose the Fisher Scientifics, Fisherbrand Cat. No. 08-0801 Lot 307, Gram staining kit was used. The staining process started with a small droplet of DI water deposited on a Fisherbrands slide. Using a wire loop, sterilized in a cinerator, a small colony sample was smeared on the DI water droplet. The samples were later passed

through a flame produced by 95% ethanol to evaporate any liquid residue remaining on the slide permanently fixing bacteria samples to the slide. Digital pictures were cataloged after each analysis and subsequent attempts were made to classify the bacteria.

3.8 Statistical Analysis

Statistical analysis was done using Excel's data analysis statistical tool packet. The statistical analysis of this project was restricted by the number of samples collected from each site. The rejection quotient test, Q test, to determine outliers from each sample was used. Outlier value is a term that was cautiously used in this experiment as the interactions of microorganisms present in the bioaerosol and web's chemical matrix are not clearly understood. Also, the age of the web may influence counts. If a collected web had been exposed for longer or significantly less than the assumed 24 hours it is possible that the counts would be affected, therefore causing outliers. Student t test, two-tailed assuming equal variances, with 95 % confidence interval was done for the samples from the parking garage to determine the effects of seasonal variations and for the WWTF samples to determine the existence of a saturation point. Box plots were also created to compare the spread of the samples and determine if they were correlated.

3.9 Summary

The research objective of this experiment had no precedent in peer-reviewed literature. After trial and error, it was proved that microscopy slides could be used as a collection instrument. The use of CG microscopy slides as silk collection apparatus permitted sterile collection of spider webs with minimal interference from background bioaerosols, allowed for in situ microscopy analysis of observed growth, and provided a

uniform quantification area. Quantification of colonies did not follow the normally accepted standard plate count or flow cytometry protocols. Independent colonies observed underneath the slides were attributed to the silks while growth outside the slide was attributed to background interference. The selected sampling areas addressed research concepts of interest such as, solar radiation effects on viability of microorganisms and impact of vehicular traffic on web collection capabilities.

IV. Results and Analysis

4.1 Overview

Results from four sampling locations suggest that spider webs could be used as natural passive bioaerosol collectors. Spider silks are ubiquitous in locations where physical and environmental conditions can affect the dispersion dynamics of bioaerosols and the collection properties of spider silks. The observed mean counts per slide (CPS) varied between locations; perhaps indicative of bioaerosol composition, cell viability, environmental conditions, or webs collection properties. The life cycle of spiders dictated that samples be collected no later than October 15, 2008. On account of the collection time constraint, only the parking garage was sampled on two separate occasions. The lack of repetitive samples and high variability in field environmental conditions limited an adequate statistical comparison between the locations. Nevertheless, using the parking garage sample sets, the student t test was used to obtain insight pertaining webs' collection capabilities under different seasonal conditions. The student t test was also used to determine a possible web collection saturation point using the sample sets from the WWTF. And finally, a rough site comparison on the recovered mean population was done using JBSP as control set. Results of microbial growth are expressed as average CPS (± standard error of the mean).

4.2 Observed Growth

Silks collected from four locations generated bacterial and fungal growth. In this study, 72 hours of growth revealed what was considered the most accurate enumeration data. After 72 hours, a white residue for NA samples and a dark discoloration for SDA

samples overwhelmed the silks and the area under the slide restricting further accurate enumeration. For bacteria, the garden center set yielded the highest CPS average while the summer parking garage set produced the lowest, Table 6. For fungi, the WWTF sets produced the highest CPS average while JBSP set produced the lowest, Table 6. Bacteria CPS averages were higher than fungi in all trials except the summer parking garage and WWTF set. These sets may had been influenced by pollution from increased vehicular traffic, dispersion dynamics, and cell desiccation due to higher temperatures. It is likely that these deviations are the result of pollution and dispersion effects since temperature conditions among the other sites were comparable.

Table 6. Average colony per slide (CPS) after 72-hours of growth

	Average Count 72-Hour (NA)	Average Count 72-Hour (SDA)
State Park	11.4 ± 3.5	5.2 ±1.8
Garage (Summer)	6.1 ± 2.5	9.5 ± 2.2
Garage (Fall)	16.1 ± 3.8	14.3 ± 4.0
Garden Center	31.8 ± 9.9	11.8 ± 2.6
WWTF	18.2 ± 1.9	23.0 ± 2.5
WWTF (500 m)	23.3 ± 2.3	16.5 ± 3.2

When all samples are considered simultaneously, bacteria counts are higher than fungi, 17.03 ± 2.44 CPS to 11.7 ± 1.32 CPS respectively, which is representative of the higher bacteria concentration in bioaerosols.

Fig. 14 & 15 show box plots for each location and growth medium. Each box plot was generated from the independent samples collected from each site. The CPS spread, width of the box, appears consistently higher for fungi species than bacteria. This may represent a more uniform silk collection property for bacteria than fungi.

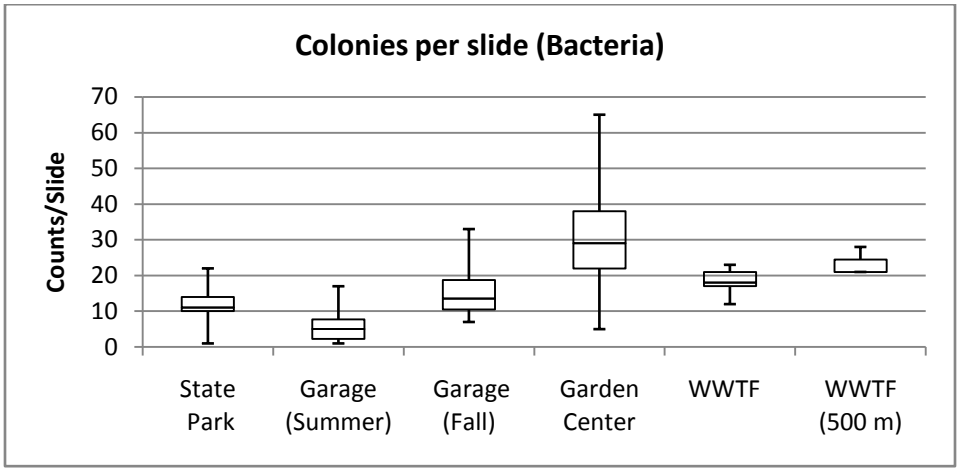

Figure 14. Colony per slide Spread (Bacteria)

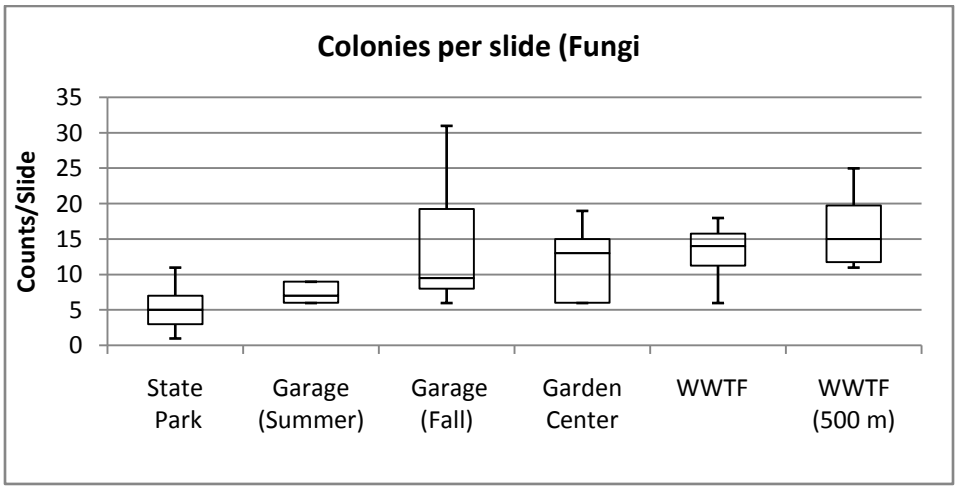

Figure 15. Colony per slide Spread (Fungi)

The implied collection uniformity could be a direct effect of microorganisms' size, viability, and/or dispersion dynamics favoring bacteria over fungi species. Stokes

law indicates that larger microorganisms, such as fungi spores, would be affected by gravitational settling easier than smaller microorganisms such as bacteria [29]. This fact, in turn, suggests that bacteria may remain airborne for longer time, favoring webs surface deposition. Microorganisms tend to have a negative charge distribution at neutral pH [29]. If bacteria species posses a stronger dipole moment it could also favor interaction via van der Walls Forces with spider silks. The differences in CPS counts between the sites should not be strictly considered a silk performance parameter. These count variations could also be influenced by deviations in bioaerosol concentration, environmental factors, and human activities in or near the sampling areas. The performance parameters of spider silks regarding the collection of microorganisms could not be defined without insight on the true bioaerosol speciation and concentration.

4.3 Site Comparison

A personal communication with Dr. Brent Opell, October 18, 2008, indicated that cribellar spiders are not indigenous to the Ohio Valley. This information focused this study on one type of silks, escribellar. Assuming that differences in webs' protein composition as a result of deviations in spider diets are negligible, a comparison of the webs' mean collection capacity was possible. The two-sided student t test, equation 1,

$$T_{n1+n2-2} = \frac{(\bar{X}_1 - \bar{X}_2) - (\mu_1 - \mu_2)}{\sqrt{(S_p^2 (\frac{1}{n_1} + \frac{1}{n_2})}} \quad (1)$$

with a 95% confidence interval was used assuming normality, equal variance, and independence. JBSP was selected as control since this location was the least disturbed by

launching sources, vehicular traffic, and SR. For both growth mediums, NA and SDA, the same hypothesis test was used

$$H_0: \mu_1 = \mu_2$$
$$H_1: \mu_1 \neq \mu_2$$

Where the mean values represented the average number of colonies per slide recovered at each site. The P-value approach was used to determine the level of significance of the null hypothesis. The P-value is the probability that the test statistic (t_o) will take the observed value given that the null hypothesis is true [33]. The average webs' collection capacity for NA samples appears to be equal, Table 7. The highest P-values corresponds to the parking garage samples, p = 0.25 for the summer set and p = 0.39 for the fall set, Table 7.

Table 7. Inference on the Mean Colony per slide count for Bacteria Samples using JBSP as Control

	Parking Garage (August)	Parking Garage (October)	Garden Center	WWTF	WWTF (500 m)
t statistic	1.239	-0.899	-1.9389	-1.695	-2.376
Mean	6.167	16.167	31.8	18.2	23.333
Two-tailed Value	2.262	2.262	2.306	2.306	2.447
H_0 Inference	Do not reject H_0	Do not reject H_0	Do not reject H_0	Do not reject H_0	Do not reject H_0
P value	0.247	0.392	0.088	0.129	0.055

JBSP and the parking garage sets had the lowest CPS averages. Shielding from SR, by tree canopies or floor ceilings, is the only physical commonality between these sites. However, reduced exposure to SR is likely not the reason for the reduced number in CPS average. Tree canopies may inhibit microbial dispersion and deposition rates; therefore, reducing the amount of microbes available for collection, while pollutants present in

Dayton's atmosphere, as a result of increase traffic, may reduce cell viability. The garden center and the WWTF samples had smaller P-values (correlation with JBSP). The garden center and $WWTF_{(500\ m)}$ P-values, $p = 0.08$ and $p = 0.055$ respectively, are close to the rejection threshold $\alpha = 0.05$. These areas differ from JBSP in that they were directly exposed to turbulent diffusion and had strong launching sources in the vicinity. Webs' collection properties seem consistent for bacteria and deviations in CPS average are probably the result of cell viability and dispersion dynamics not limitations on the webs' collection properties. For SDA, Table 8, the P-values were low for all locations with two sets, both from the WWTF, falling into the rejection threshold. These low P-values suggest that the webs' mean collection capacity for fungi species is highly variable and may not follow bacteria collection dynamics.

Table 8. Inference on the Mean Colony per slide Count for SDA Samples using JBSP as Control

	Parking Garage (August)	Parking Garage (October)	Garden Center	WWTF	WWTF (500 m)
t statistic	-1.469	-1.901	-2.089	-2.539	-3.201
Mean	9.500	14.333	11.8	13	16.500
Two-tailed Value	2.262	2.262	2.306	2.365	2.365
H_0 Inference	Do not reject H_0	Do not reject H_0	Do not reject H_0	Reject H_0	Reject H_0
P value	0.176	0.090	0.070	0.039	0.015

Environmental factors other than RH humidity and SR seemed to have a negligible effect on CPS averages. Additional information pertaining to the bioaerosol composition and concentration in the areas studied is needed. A point that must be highlighted is that microbial growth was observed in samples from all sites, which was

the primary objective of this research. This comparison brings to attention the need for further research into the possible selectivity of spider webs for bacteria. The probability of type II error (β), or false negative, was considerable given the low number of samples collected at each site.

4.4 Relative Humidity

Environmental factors may have affected the collection capacity of the silks. Percent RH humidity is a known factor that interacts with glycoproteins contained within the silks' nodes therefore affecting webs' adhesive properties [35]. Peer-reviewed literature regarding this topic focused on nodded cribellar silks not, escribellar. A private communication with Dr. Brent Opell, December 17, 2008, suggested that RH changes may affect nodes in escribellar silks in a similar mechanical pathway as nodes in cribellar silks are affected.

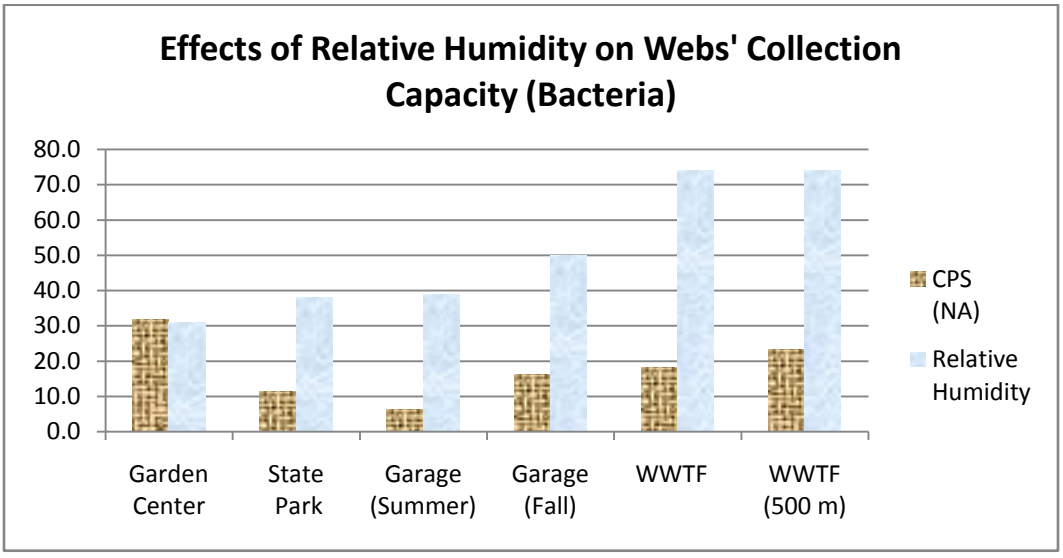

Figure 16. Impact of RH on number of colonies recovered (Bacteria)

Fig. 16 shows a comparison of daily average RH and CPS averages per location for bacteria. Relative humidity data were obtained from the National Weather Service in Wilmington, Ohio. Increases in percent RH appeared to correlate with increases in CPS averages except for the JBSP and parking garage summer set. Other factors, like pollution or microbial dispersion, may have had a stronger impact at these locations.

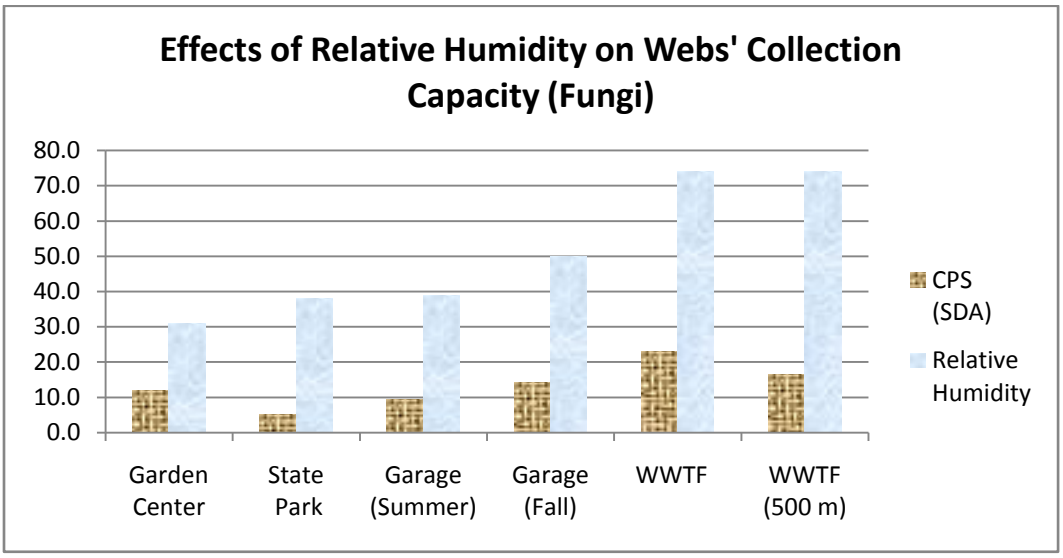

Figure 17. Impact of RH on number of colonies recovered (Fungi)

For fungi species, Fig. 17, increases in RH humidity also seemed to correlate with increase in average CPS averages except in JBSP and WWTF 500 m away from the main aeration basins. As previously stated, tree canopies may affect microbial dispersion in JBSP. The WWTF samples may have been influenced by the strong winds, remnants from Hurricane Ike, as well as gravitational settling for fungi spores. The correlation between increases in RH and CPS averages seemed consistent with finding by Opell and Hendricks involving cribellar silks [35].

4.5 Microscopy Study

Microscopy studies were done to classify the silks collected as well as observing microbial growth induced by silks. Extensive variations on the silks, which could be indicative of different spider species and/or deviations in spider diets, were observed. Most collected webs were suspended orb webs with node sizes between 5 and 10 micrometers. The variations in node sizes may have had a significant impact on the amount of bioaerosol collected by the silks; therefore, the number of CPS generated. The garden center's funnel webs, Fig. 9 (b) appeared to be cribellar silks Fig. 18, forming snares through a large conglomerate of fibrils.

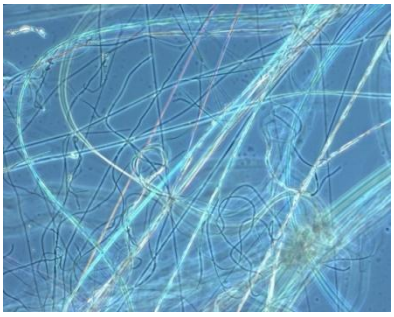

Figure 18. Garden Center Funnel Web, magnification 200 X.

Silk samples consistently showed a dark residue following the silk contour after deposition on the growth medium, Fig. 19 (a) and Fig. 19 (b). This residue often seemed to be a precursor to microbial growth, but its true composition could not be determined Fig 19 (c).

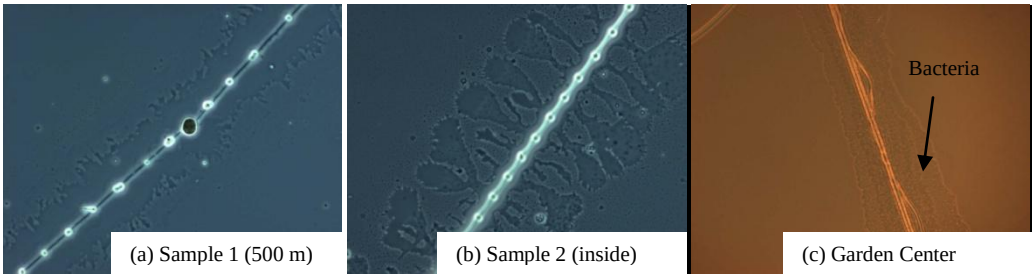

Figure 19. Residue on Webs, magnification 200 X

For fungi species, the growth was akin to bacteria. Hyphae initiated on the fibrils and formed a mycelium which later spread throughout the growth area Fig. 20.

Figure 20. Garden Center, magnification 50 X

Observed microbial growth included cocci, bacilli, and other sporulating bacteria while fungi revealed what appeared to be basidiospores, and ascospores Fig. 21.

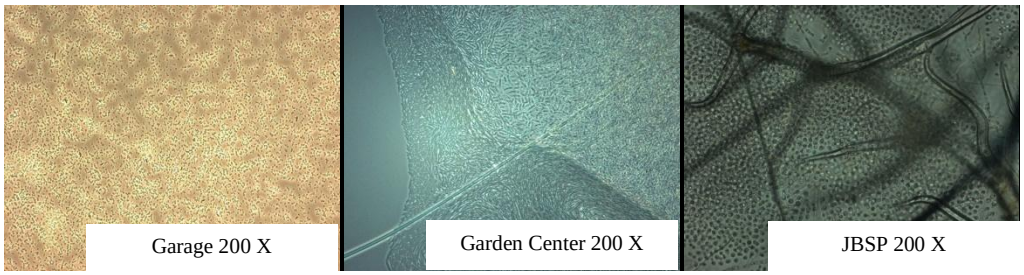

Figure 21. Example of observed Microbial Growth

These observations were done by comparing the shape and appearance of the growth with literature descriptions. The positive identification of these microorganisms requires equipment and technical expertise not presently available. These results highlighted the capability of silks to recover a wide variety of microorganisms which could possibly lead to the collection of pathogenic species. The growth could not be pinpointed to bioaerosol deposition on the silks or collected particulate. Microorganisms are known to deposit on airborne particulate [11,29]. There were instances where particulate was present on the silk and microbial growth appeared to be induced by these particulate, and other instances where no microbial growth was visible despite the presence of debris. Silks, with no sign of particulate, also induced microbial growth at random. It is likely that both particulate and bioaerosol deposition on silks induced microbial growth.

Gram staining, to an extent, confirmed the suitability of silks as passive bioaerosol collectors. The staining revealed what could be classified as Gram positive and negative bacteria, Fig. 22. This point gives credence to the belief that spider webs do not discriminate between Gram positive or Gram negative bacteria.

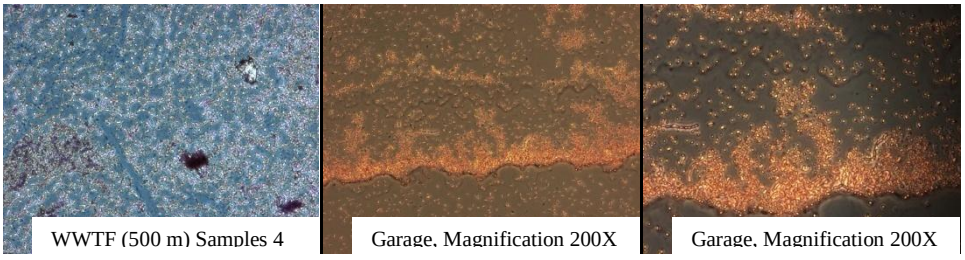

Figure 22. Gram Stains

Despite evident variations in node sizes, web's location, i.e. surface vs. suspended, and types, all samples collected produced microbial growth. The growth was not uniform across the webs fibrils. There were instances in which web fibrils did not appear to promulgate growth. This could be the result of zero gravitational settling of microorganisms, the presence of non culturable microorganism, parasitic interactions by non-culturable microorganisms, or deposition of non viable cells in those regions.

4.6 Saturation Point and Seasonal Variation

The sample sets collected from the WWTF addressed web saturation concerns as a result of a strong microbial launching source near the silks. The WWTF sets were collected from locations that were about 500 meters apart to offset any possible saturation point. A saturation point is a factor of interest when determining the suitability of spider webs as natural passive collectors. Spider silks to be a viable collector must be able to continue to collect aerosolized microorganisms in the presence of strong launching sources such as a WWTF. If the silks are saturated, they have a low limit of collection; they may be considered inadequate passive collectors. For reasons not directly attributed to web collection capabilities, the parking garage was visited on two separate occasions, one in the summer and one in the fall. Inadvertently, these sets permitted the study of webs' collection capabilities under different environmental conditions caused by seasonal changes.

WWTF

CPS counts do not indicate a web saturation point. In fact, webs appeared to collect a variable number of colony forming microorganisms regardless of the distance

from the main aeration basins, Fig. 23. For both mediums, background samples indicate a higher microbial concentration near the aeration basin, Fig. 23. For bacteria, samples collected 500 m from the aeration basin yielded the higher CPS average. For SDA, samples collected near the aeration basin yielded a higher CPS average. The perception is that microorganisms generated by the aeration process rise in an upward movement, following a conical dispersion as described by Maier [29]. In the area 500 m away from the aeration basin, gravitational settling and Brownian motion may support bacteria deposition. The student t test suggests that these samples have the same population mean with p = 0.14 for NA samples and p = 0.43 for fungi samples.

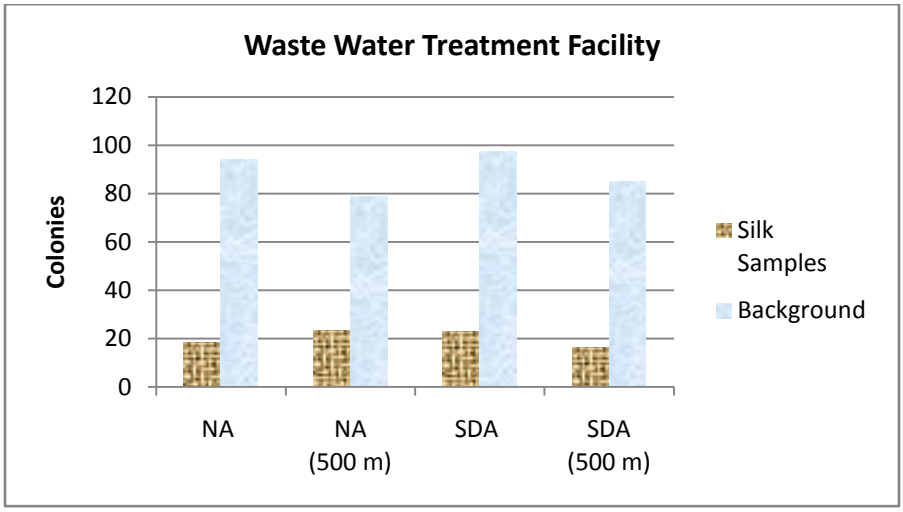

Figure 23. WWTF Average Growth Comparison given a 500 m Standoff Distance between Collection Areas

The difference in P-values is probably representative of bacteria and fungi dispersion patterns, as previously explained, not web's collection characteristics.

The average growth for samples collected near the aeration basin were 18.2 ± 1.9 (CPS) for bacteria and 23.0 ± 2.2 (CPS) for fungi. Samples collected 500 m away from the aeration basins yielded an average growth of 23.3 ± 2.3 (CPS) for bacteria and 16.6 ±2.2

(CPS) for fungi. Although, a saturation point is likely to exist, it cannot be identified with present data sets.

Parking Garage

The Fall background samples yielded a significant higher average colony count than the summer samples, Fig. 24. This is likely the consequence of changes in bioaerosol population as a result of human activity variations and increases in percent RH.

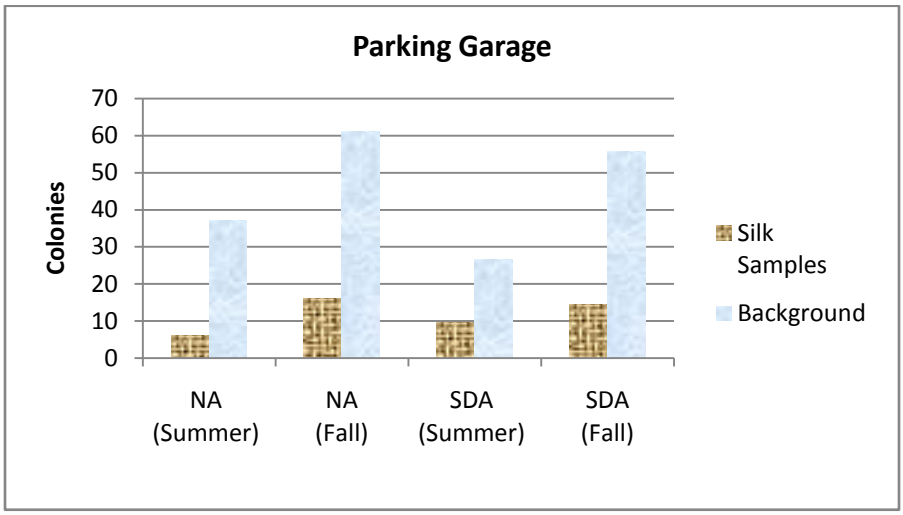

Figure 24. Seasonal Comparison using Parking Garage Samples

In the summer, the lower average count may be the result of possible cell inactivation caused by higher temperatures or DNA intrastrand dimerization caused by higher UV radiation. Increases in vehicular traffic, as a result of summer leisure activities in the City of Dayton, may had also reduced microbial populations through a number of released pollutants. The student t test suggest that the collected population means are the same, with $p = 0.053$ for bacteria and $p = 0.16$ for fungi species. The bacteria samples

are almost within the rejection threshold of $\alpha = 0.05$. The higher P-value for fungi could be indicative of fungi spores resisting the adverse effects of environmental factors as well as pollutants better than bacteria in the summer. The percent RH, which affects the hygroscopic glycoproteins in the webs, was higher in the Fall set, 50% compared to 39% in the Summer. Although, the increase in RH may increased the webs collection properties, the rise in bioaerosol concentration curtails the possibility of attributing the higher CPS average to just enhanced webs' collection properties.

4.7 Summary

Silks collected from all four locations produced microbial growth. The observed growth, as expected, was highly variable. The student t test suggested that the mean collection capability for bacteria is similar while the mean collection capability for fungi appeared to be different despite consistent sampling techniques and environmental conditions. This may be the result of different dispersion dynamics as well as bioaerosol concentration for each type of microorganisms. Gram stains showed the presence of both type of bacteria and webs did not discriminate in their collection. Changes in RH seemed to be proportional with the observed CPS averages. Statistical analysis was restricted due to the number of replicate samples collected from each location. In the future, additional replicate samples must be collected to define spider webs performance parameters.

V. Discussion

5.1 Research Question

- Can spider webs be used as passive bioaerosol collectors?
    - During this study, spider silks passively collected aerosolized microorganisms from four different locations under variable weather conditions. Fungi and bacteria species were recovered from all sampled locations, but webs collected higher amounts of bacteria than fungi. This could be indicative of higher bacterial concentrations in bioaerosols, microorganisms dispersion dynamics, cell viability, or web collection properties. The silks appeared to collect microorganisms representative of background populations.
    - This research was restricted by the number of replicate samples collected. Statistical analysis had a considerable probability of type II (β) error as a result of the limited number of samples collected.

5.2 Impact of Research

- The applications of spider webs as passive collectors are broad. The minimal cost concerning the use of webs as collectors, their ubiquitousness, and ample availability may influence future field sampling initiatives. Spider webs may also find applications in combating terrorism and proliferation. Historical signatures left by covert activities of terrorist and proliferants states may be detected and traced using spider webs. Also, webs could be used to determine post incident contamination boundaries. This study was motivated by bioterrorism threats but,

webs may also find applications in identifying signatures from chemical warfare agents (CWA) and radioactive material.

5.3 Recommendations

A sequential approach should be developed in order to define silks collection and performance parameters. Research that addresses webs' collection effectiveness, limit of collection/detection, and limitations should be a priority. A two pronged approach should be considered in the future; one that focuses on controlled experiments that define performance parameters and another that focuses on field sampling and comparisons against more sophisticated field sampling methodologies.

5.4 Suggestions for Future Research

- **Spider Webs Suitability as Bioaerosol Collector**
  - A limit of detection/collection must be defined. All samples produced microbial growth; however, the amount and speciation recovered by the webs in relation to the true bioaerosol composition was not determined in this study. Field experiments in which signatures (species) and densities recovered by spider webs are statistically compared to signatures recovered by more sophisticated field sampling technologies, such as Anderson samplers, should be conducted. This comparison would provide insight into webs' collection capacity and possible discriminating factors.
  - Environmental factors may influence webs' collection properties. Relative humidity, for example, has been proven to affect silk adhesive properties by interacting with glycoproteins present on the webs [35]. Other environmental

factors, such as solar radiation and temperature, may also affect physical and molecular properties within the silks that could result in variations in adhesive capacity. Controlled experiments should be conducted where adhesive properties are correlated to changes in environmental conditions following a factorial approach. The size and concentration of microorganisms present in bioaerosols may permit their collection despite variations in environmental conditions. However, these studies may help define webs' performance parameters and efficacy as a bioaerosol collector.

- Microorganisms must be positively identified to determine possible collection biases. In this study, morphologic comparisons between species observed in background samples and species observed in silk samples suggested that webs collected bioaerosols in their true composition without discriminating family or genera. The use of flow cytometry, serial dilutions, and polymerase chain reaction (PCR) in future studies is highly encouraged. Flow cytometry could help in determining size, phenotype, and complexity of cells present in heterogeneous samples. Serial dilutions could establish quantitative parameters while PCR or other molecular analysis could positively identify, at a minimum, the genus of the microorganisms.
- A saturation point should be sought and determined. In the field, silks' surface area may become saturated by the constant deposition of particulates and microorganisms as a result of strong launching sources near a sampling area or protracted exposure to bioaerosols. A saturation point may restrict the use

of old webs in the absence of newly woven silks or in the presence of strong microbial launching sources.

- Fig. 25 depicts a road map that could guide future qualitative and quantitative studies using spider webs as passive bioaerosol collectors.

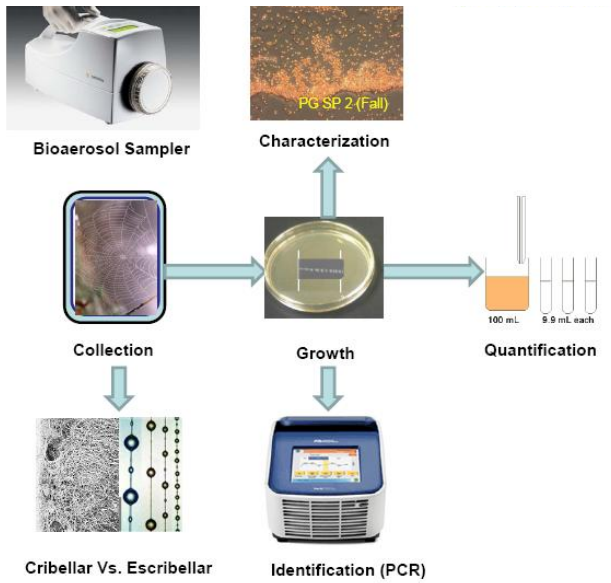

Figure 25. Example Road Map for Future Research Involving Bioaerosol Collection

- Silks could be collected using an open instrument such as a ring with its outer contour covered with double-sided adhesive material. The instrument could be thrust into a silk forcing it to become embedded on to the collection instrument. Structural integrity should be unaffected. Using this collection methodology permits the storage of silks for future studies; therefore removing the collection time constraint. A key aspect in any future research is to establish a base of comparison. A base comparison for microorganisms could be obtained by using one of a number of commercial bioaerosol samplers. The use of molecular technologies would overcome underestimation constraints inherent in culture

techniques and quantitative procedures such as serial dilutions could provide critical insights into the collection capacity of the silks.

- **Spider Webs Suitability as Chemical Agent Collectors.**
  - This study focused on the collection of aerosolized microorganisms but, signatures of chemical agents released in their liquid or gaseous forms may also be detected using webs. Hose et al. showed the suitability of silks as a passive collector of chemical pollutants [20]. It is possible that Hose et al.'s findings and methodology could be applied when searching for signatures generated by chemical warfare agents (CWA).
  - Silks are composed predominantly of proteins. The proteins present in the silks could interact with chemical agents to the extent of forming new chemical compounds, dissociating or breaking, or continuous collection while maintaining their full chemical, molecular, and structural integrity. Control reactions should be conducted to determine the effects of chemical groups on silks and their remaining signatures.
  - If silk preservation is desired the collection methodology defined for bioaerosols should be followed. However, silks may be collected using unsterilized media with negligible impact on the analysis. Silks could be analyzed using destructive or non destructive methods. If an aliquot must be prepared silk digestion using nitric acid or hydrofluoric acids had been proven effective in the past [2,20].
  - Chemical agent signatures could be identified using spectroscopic or chromatographic methods. Fourier transform infrared (FTIR) spectroscopy,

Gas Chromatography-Mass Spectroscopy (GS-MS), Raman spectroscopy, and nuclear magnetic resonance (NMR) are some of the technologies currently used when searching for chemical warfare agent signatures. These technologies take advantage of chemical compound properties such as charge, spin, and vibrational characteristics. The chemical properties of the compounds of interest must be understood before selecting a technology.

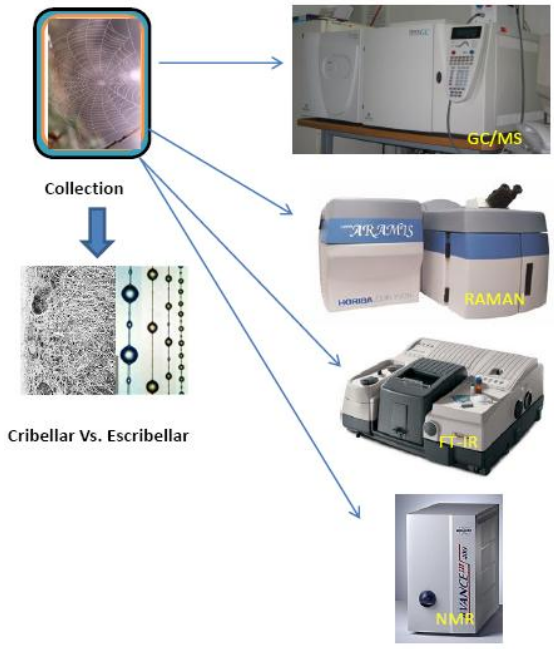

Figure 26. Example Road Map for the Analysis of Chemical Agents

- Fig. 26 depicts a road map that may be used to guide future initiatives involving chemical substances. Spider silks molecular composition will generate signals characteristics of their protein matrix. However, the signature produced by CWA should be visible and discernable. When working with chemical agents, signatures of interest are normally generated by P-CH3, P-CH2CH3 bonds. More specifically, methylphosphonic acid, a degradation product of VX and sarin, ethyl

methylphosphonic acid, degradation product of VX, and isopropyl methylphosphonic acid, a degradation product of sarin. Chemical compounds containing some of these groups could be used as surrogate for the CWA in future studies.

- **Radioactive Material**
  - There are two primary threats involving nuclear material, a nuclear detonation and dirty bombs. A dirty bomb, also known as radiological dispersal device (RDD), combines conventional explosives with radioactive material to contaminate a region with radioisotopes. This threat is not expected to cause large number of casualties but, the psychological and economic impact, due to expensive clean-up, would be severe. During RDD events, silks could find limited application in defining contamination boundaries.
  - A nuclear detonation requires weapons grade fissile material primarily uranium-235 (^{235}U) or plutonium-239 (^{239}Pu). Weapons grade fissile material is normally obtained from uranium-238 (U-238). The fissioning of an atom of uranium-235 in a nuclear reactor produces two to three neutrons. These neutrons can be absorbed by uranium-238 to produce plutonium-239 and other isotopes Equation 2.

$$\boldsymbol{n + {}^{238}_{92}U \rightarrow {}^{239}_{92}U \rightarrow {}^{239}_{93}Np + e^{-} + \overline{\nu}_e} \qquad \textbf{(2)}$$
$$\boldsymbol{{}^{239}_{93}Np \rightarrow {}^{239}_{94}Pu + e^{-} + \overline{\nu}_e}$$

  - Natural uranium is predominantly composed of U-238 (99.28%) and U-235 (0.71%). U-235 is obtained by enriching uranium ore, separating U-235 from U-238, based on the isotopes mass differences. With technological advances

the enriching process is becoming more efficient. Spider webs may find limited application in situations when sampling technologies are inaccessible or not permitted, i.e. during inspections. Some isotopes might migrate away from enrichment confines and deposit on silks. Isotopes emit characteristics signal at energy channels that have been thoroughly studied. If an isotope associated with U-238 and Th-232 decay chains is detected it could be indicative of covert enrichment activities.

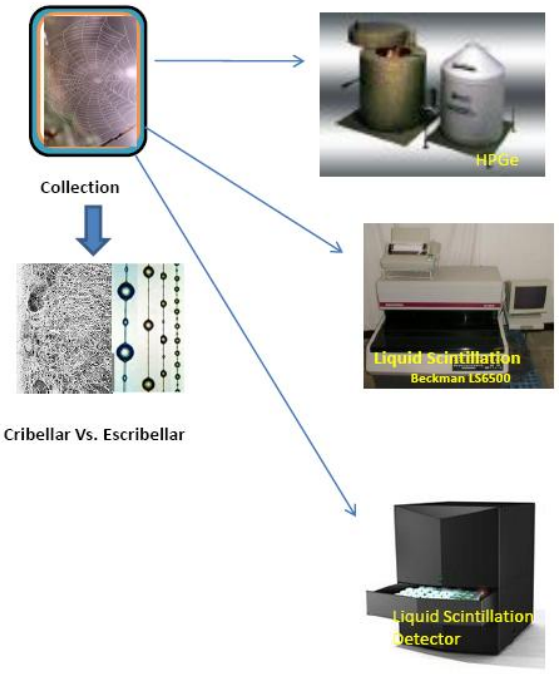

Figure 27. Example Road Map for the Analysis of Signatures from Radioactive Material

- Fig. 27 shows a road map that could be used to study the suitability of silks as isotope collectors. Signatures of interest involve alpha (α), beta (β), and gamma (γ) decaying isotopes associated with nuclear material. It is highly recommended that silks be analyzed for signatures generated by radioactive fallout present in

atmospheric air. Also, webs may be exposed to radioactive material and analyzed for spikes in background signatures or new signatures corresponding to the exposed material. Collection would follow a pattern similar to what was previously explained. High purity germanium (HPGe) detectors and liquid scintillation detectors are widely used when working with α, β, and γ signature emitters

5.5 Conclusion

This proof of concept experiment examined the suitability of spider webs as passive bioaerosol collectors. The high cost of current technologies employed in the detection of aerosolized pathogens, their restriction in determining post incident contamination boundaries due to their fixed location, and their limitation in providing post incidents historical data motivated this research. Spider webs were considered a suitable proxy for these technologies since webs are ubiquitous in most environments and geographical settings, their collection is highly inexpensive, and their constant regeneration allows the webs to become, in essence, new sampling devices every 24 hours. For spider silks to be considered suitable bioaerosol collectors they had to satisfy three basic parameters; (1) indiscriminate collection of microorganisms of different species and sizes, (2) uninterrupted collection under different environmental conditions, and (3) saturation avoidance in the presence of strong launching sources. It is cautiously concluded that spider webs satisfied these preconditions. Quantitative and qualitative analysis was not the primary goal of this experiment. Instead, the desire was to prove if silks could collect aerosolized microorganisms and preliminary define some of the webs

collection characteristics. The target population of this study were heterogeneous bacteria and fungi communities collected from four locations near Wright Patterson Air Force Base; a state park, a waste water treatment facility, a garden center, and a parking garage. Current results suggest that silks collected microorganisms without discrimination based on species, genera, or sizes. Variability in the enumeration of targeted microorganisms was observed throughout the experiment. The variability in the number of colonies is likely the result of human activities in the vicinity of the sampling sites, environmental factors such as solar radiation, and dispersion dynamics of aerosolized microorganisms, not web collection properties. Temporal and climate changes may have altered physical and molecular properties of the silks. After a private conversation with subject matter experts, it is concluded that; although temporal and climate changes may have altered silks' mechanical and molecular properties, these changes should have resulted in negligible impact on the collection of the diminutive aerosolized microorganisms. The existence of a collection saturation point was explored using the samples collected from the WWTF. A saturation point could not be confirmed with current results, but it is believed that if it exists, this point is an extreme number of particulate and microorganisms that may or may not mask the signature left by a pathogen. A limited number of collected silks generated a large number of colonies. This extensive growth could have been the result of aged silks that continued to collect bioaerosols after being abandoned. Opell and Schwend determined that silks retained between 70% and 90% of their adhesive properties after aged under laboratory conditions for up to three months [36]. It is plausible that, under field conditions, silk also retained

their adhesive properties despite aging; therefore, producing a large number of colonies and signatures when compared to recently woven silks.

In the future, additional experiments should contain a base reference in which signatures obtained from silks could be statistically compared to other methodologies. The principles guiding this experiment should not be limited to the collection of bioaerosols. It should be expanded to areas involving signatures generated by radioactive material associated with enrichment activities (alpha, gamma or beta decay isotopes) and precursors used by or degradation compounds generated by chemical warfare agents. Defining silk collection parameters should be intently pursued in order to establish limits of collection/detection and identifying possible limiting factors. At this initial stage, spider webs appear to be adequate bioaerosol collectors. The present security situation faced by the international community requires not only the use of the most advanced technologies, but also the use of other inexpensive and unorthodox methods that prove adequate in the detection of signatures of interest.

Appendix A. Microbial Variability with Elevation Increases

The AMPCO parking garage located in the city of Dayton, Ohio revealed a peculiar variability in backgrounds samples with changes in elevation. Background samples, as previously stated, were collected by gravitaty impaction. Petri dishes containing Nutrient Agar (NA) and Sabouraud's Dextrose Agar (SDA) were placed on the ledge of the parking garage facing 3rd St. on the second, fifth and eight floors. It was noticed that for both growth mediums the colonies formed decreased with increases in elevation. To further explore this finding, a visit to the parking on October 25, 2008 focused on background collection. On this occasion all floors within the parking garage were sampled, with the exception of the fifth floor. This new study validated the original observations, Fig. 28 & 29. It seems that both types of microorganisms reach a point of equilibrium between the seventh and eighth floors. Additional samples at higher altitudes are recommended to confirm if microbial concentrations continue to vary with altitude variations.

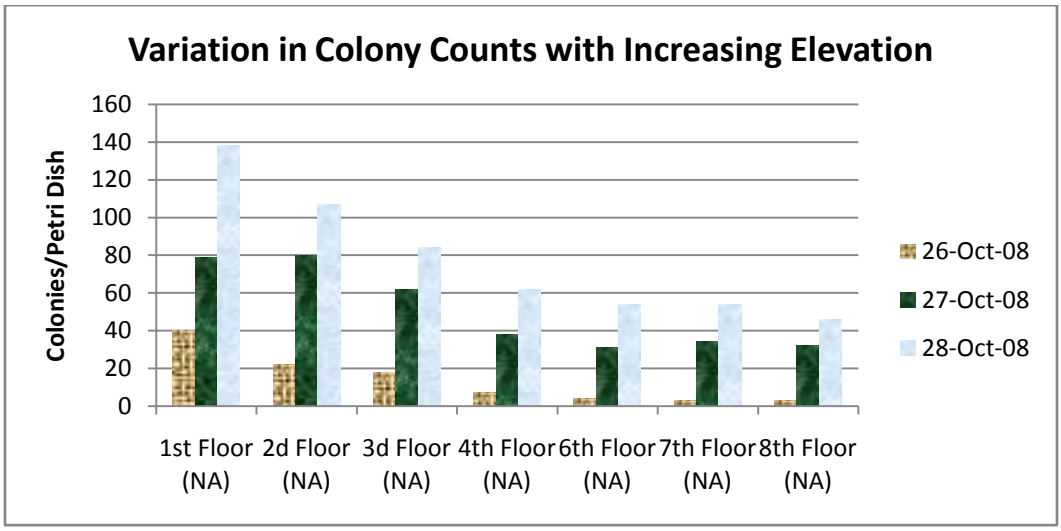

Figure 28. Bacteria Variation in CFU with Elevation Increases

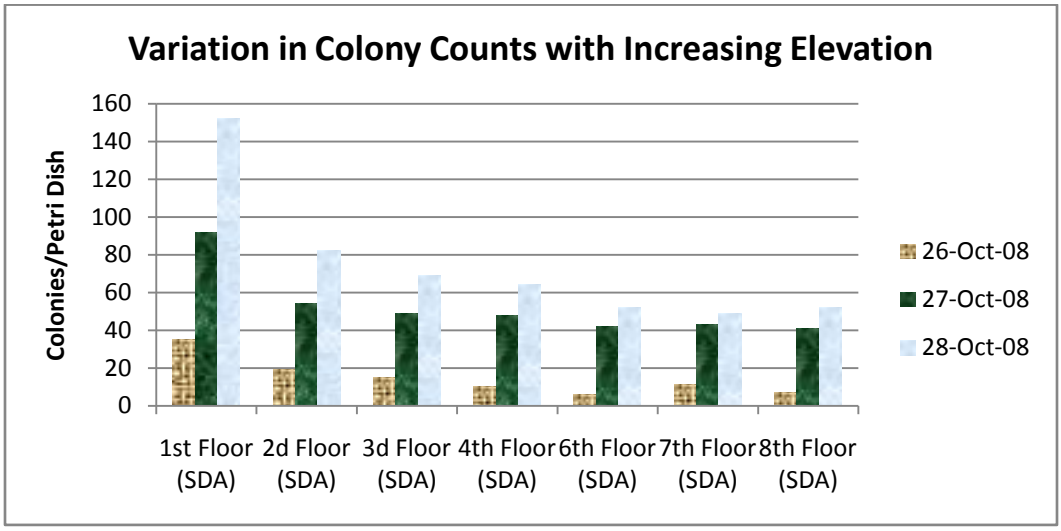

Figure 29. Fungi Variation in CFU with Elevation Increases

This finding may be of interest to government agencies charged with BioWatch sensor emplacement. Bioterrorism planning scenarios often involve the assumption of an aerosolized pathogen plume moving into a city. This scenario often assumes an aerosolized release source and that the pathogen movement will be from top to bottom. For this reason, sensors are normally deployed at high altitudes. If instead of an airborne release, a surface release occurs, sensors located at high altitudes may miss the signature; leaving the city vulnerable to the pathogen and response agencies unaware of the incident. This observation should be further explored to determine if the true microbial concentration at different altitudes is statistically different and if it may hinder detection and identification.

Appendix B. Daily Growth

The most prevalent microbial launching sources around the sampling areas were believed to be agricultural activities. A gram of soil is known to contain about 10^{10} bacteria cells and between 10^4 to 10^6 fungi CFUs [29]. Daily microbial growth seemed to follow the four steps associated with binary cell division for bacteria. Immediately after deposition on the growth medium there was evidence to suggest a lag phase (discoloration and perturbations in the growth medium) followed by exponential growth < 24-hours (microorganisms dependant). After 48-hours the growth seemed to enter a stationary phase which for some microorganisms lasted until the completion of the observation period of five days, a death phase could not be confirmed.

Table 9. Daily Growth (Bacteria)

	Average Count 48-Hour (CPS)	Average Count 72-Hour (CPS)	% Increase
State Park	8.0 ± 2.4	11.4 ± 3.5	29.8
Garage (Summer)	4.5 ± 2.4	6.1 ± 2.5	26.2
Garage (Fall)	11.5 ± 2.7	16.1 ± 3.8	28.6
Garden Center	19.0 ± 6.2	31.8 ± 9.9	40.3
WWTF	10.6 ± 1.8	18.2 ± 1.9	41.8
WWTF (500 m)	8.7 ± 0.9	23.3 ± 2.3	62.7

Table 9 shows CPS averages for bacteria species. The daily growth for 24-hours was not documented as there were instances where independent colonies could not be identified despite the presence of discoloration and perturbations. The highest increases in CPS

were observed in the garden center and WWTF sets. These areas were expected to yield the highest microbial growth as a result of strong microbial launching sources in their vicinity. The percentage increase in CPS seemed consistent among the sampled areas except for the WWTF samples collected 500 m away from the aeration basins. Fungi have variable growth rates and, as a group, they are not as capable of rapid growth as bacteria [29].

Table 10. Daily Growth (Fungi)

	Average Count 48-Hour (CPS)	Average Count 72-Hour (CPS)	% Increase
State Park	5.0 ± 1.5	5.2 ±1.8	3.8
Garage (Summer)	8.5 ± 1.9	9.5 ± 2.2	10.5
Garage (Fall)	10.7 ± 2.6	14.3 ± 4.0	25.6
Garden Center	10.6 ± 3.0	11.8 ± 2.6	10.2
WWTF	8.8 ± 2.7	23.0 ± 2.5	61.7
WWTF (500 m)	15.5 ± 2.2	16.5 ± 3.2	6.1

In comparison with bacteria samples, fungi samples demonstrated a consistent lower CPS. As mentioned above, this could be the result of the lower concentrations of fungi species in the environment or dispersion dynamics. The percentage increase for fungi is significantly lower than bacteria species, Table 10. This is indicative of the fungi's slower growth rate. It is believed that several different species were collected during this study. The variations in percentage increase among sampling sites and growth mediums is representative of the heterogeneous sampling environment.

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Vita

Major Daniel I. Mattei graduated from Luis Muñoz Marin High School in Yauco, Puerto Rico. He entered undergraduate studies at the University of Puerto Rico (Mayagüez) in 1994. He graduated in 1998 with a bachelor degree in analytical chemistry. While attending school, he joined the Army's Reserve Officer Training Corp (ROTC) in 1995 eventually receiving a commission as a second lieutenant in 1998 in the Quartermaster Corp. He attended the Defense Language Institute, Lackland Air Force Base, Texas, and Quartermaster Officer Basic Course, Virginia between 1998 and 1999.

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14. ABSTRACT

In this experiment, spider webs demonstrated their suitability as passive bioaerosol collectors. For spider webs to be considered suitable passive collectors webs had to satisfy three basic conditions; (1) collection of microorganisms without discrimination based on species or size, (2) collection under variable environmental conditions, and (3) saturation avoidance in the presence of strong microbial launching sources. Four field sampling locations were used, a waste water treatment facility, a commercial garden center, a secluded state park area, and a parking garage. Microscopy cover glass slides were used as the collection instrument. The methodology assured sterility during collection and promoted in situ microbial growth and observation which were important aspects in this study. All collected spider webs revealed microbial growth from both bacteria and fungi species.

This experiment paved the way for future use of webs as passive collectors of biological warfare agents and chemical warfare agents.

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