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A novel periodic sampling method to assess airborne bacteria populations

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entitled

A Novel, Periodic Sampling Method to Assess Airborne Bacteria Populations

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements

for the Master of Science Degree in Biology

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An Abstract of

A Novel, Periodic Sampling Method
to Assess Airborne Bacteria Populations

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Prolonged aerosolization of bacteria can result in a significant public health threat. However, the community composition of aerosolized bacteria, in indoor environments for example, is often poorly characterized. This is because traditional air collection is limited to a one-time, short duration sampling (e.g. 10-60 minutes), during which an arguably non-representative air sample is often collected. To address this shortcoming, a periodic air sampling protocol was developed in an effort to sample in a more representative portion of airborne bacteria.

In contrast with typical impaction onto an agar surface, the novel, periodic sampling method (i) operates periodically (e.g. 10 min h^{-1} for 48 h) over an extended period (e.g. multiple days), and (ii) impacts bacteria onto nitrocellulose membranes (0.45 μm pore size) overlaid onto 60 mM peptone-phosphate agar. Following sampling, membranes can be removed from the agar and analyzed to determine cell counts on general- or organism-specific media, or assayed with molecular tools to generate genetic fingerprints or to detect genes of interests.

In preliminary tests of several agar options, 60 mM phosphate buffered agar was found to most effectively facilitate survival of *Escherichia coli* and *Staphylococcus aureus* inoculated onto nitrocellulose membranes. Sampling under controlled conditions followed by DNA isolation and denaturing gradient gel electrophoresis analysis showed that despite equivalent total sampling durations (one, 60 min sampling vs. 10 min h^{-1} for 6 h), bacteria communities collected using composite sampling were better characterized with respect to the number of sampled communities than those collected using traditional methods ($p \leq 0.05$). Composite sampling also detected significantly higher bacteria numbers versus those generated from the traditional sampling method ($p \leq 0.05$).

Compared with traditional sampling, composite air sampling provided a more comprehensive characterization of airborne bacteria. Additionally, greater downstream analytical flexibility can be of great importance for characterizing environments exhibiting dynamic air quality.

To my teacher, my advisor - **Dr. Von Sigler** - in Sanskrit we have a saying that if both God and your teacher were to appear in front of you, you should bow down to your teacher who introduced God to you.

To my strength - **My Grandma** - who never sheltered me from dangers, but taught me to be fearless when facing them.

To my idols - **My Parents** - for their undying faith in me and constant support in everything I have pursued and want to pursue.

To my *corazon* - **Juan** - for being my support through every right or wrong decision I've made and never letting me fall or lose hope.

To my angel, my sister, my best friend - **Bern** - I must have done something extraordinarily amazing in life to have you by my side.

To my brother, my dada - **Kanak** - you asked me, when I was 9, who my favorite person in the family was. I never answered your question because I was too busy eating the buttered toast Dimmana made for us. The answer has always been YOU, it will always be YOU.

To everyone who made my stay in Toledo worthwhile, never let me get enough sleep and always woke me up with a cup of hot tea.

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CHAPTER 1

Introduction

Bioaerosols are defined as airborne particles that include living organisms or materials released from living organisms (ACGIH, 1999), and are usually of microbial, animal or plant origin, varying in size from 0.01 to 100 μm (Fabian *et al*., 2005). Bioaerosols can be composed of either live or dead, pathogenic or non-pathogenic bacteria, as well as viruses, fungi, peptidoglycans and pollen (Douwes *et al.*, 2003). Interest in indoor bioaerosol exposure has increased over the past decades primarily because major public health impacts have been associated with exposures to biological aerosols in both residential and occupational settings (Douwes *et al.*, 2003).

Bioaerosols in indoor environments can originate either from outdoor environments, sources within the building (occupants and their activities) or microbial growth on building materials (Fabian *et al*., 2005). Several studies have shown that exposures to biological agents can be abundant in indoor environments. For example, nosocomial infections pose major public health concerns for both hospital staff as well as patients and are often the result of exposure to bioaerosols (Gilbert *et al*., 2010). It has been reported that bioaerosols may account for 10-20% of all endemic nosocomial infections (Brachman 1970). Respiratory illnesses and lung diseases are among the most widely

studied bioaerosol-associated health effects (Douwes *et al.*, 2003), while others include contagious infectious diseases, acute toxic effects, allergies and cancer (Douwes *et al.*, 2003; Stetzenbach *et al.*, 2004). While several bacteria such as *Staphylococcus aureus, Sphingomonas paucimobilis* and *Pseudomonas aeruginosa* are known to cause the majority of nosocomial infections (Gilbert *et al*., 2010), few studies have been proposed to show the ecology and bacterial diversity of bioaerosols in such hospital environments (McBain *et al.*, 2003), especially over a period of days. People within residential units, especially infants, spend 90% of their time in indoor environments (Klepeis *et al.*, 2001). Hence, it is crucial to sample and assay such indoor environments over time and changing environmental conditions in order to reduce the risk posed by airborne infectious agents.

Although the importance of bioaerosol exposure to human health has been established, the data explaining the dynamics of bioaerosols results largely from methodology that is limiting with regard to detecting airborne microorganisms (Douwes *et al*., 2003). One popular method of sampling airborne microbes utilizes impaction, in which a vacuum pump is used to pull air through a manifold containing numerous holes (0.25 mm diameter), followed by deposition (impaction) of the airborne microbes onto a nutrient agar plate that is positioned below the manifold. Traditionally, sampling is performed for approximately ten minutes at an air flow rate of 28.3 L of air min^{-1} (ACGIH, 1999). In some situations, this sampling duration is limiting, as the densities of airborne microbes is usually low or varies over time depending on several factors including the source, environmental changes, principles of gravity and turbulence (Lin *et al*., 1999). Additionally, airborne microbial densities are not homogeneous (Zollinger *et*

al., 2005) and fluctuate based on the sampling location, changing environmental factors and the level of human and/or animal activity. Therefore, traditional sampling times can result in non-representative characterizations of microbial load and community composition. Extending the sampling time to encompass a more representative time-frame might appear a viable option to overcome these limitations. However, sampling times exceeding ten minutes often cause overloading of microorganisms (Johnson *et al*. 2008) and desiccation of the media that reduces overall sampling efficiency and viability of microorganisms on the agar (Hensel *et al.*, 1995).

It has been recognized that airborne microorganisms can vary over several orders of magnitude with time and depending on the location. For example, in residential indoor environments, densities of aerosolized bacteria can range between 35 and 22,000 CFU m^{-3} (Lin *et al.*, 1999; Lai *et al.*, 2003). Therefore, increased sampling times can most likely improve sampling representation, decrease variability between samples, and more accurately assess exposure risk, as compared to traditional short term sampling events (Lee *et al*., 2006; Lin *et al.*, 1999; Pasanen, 2001). To effectively collect airborne microbes using sampling times approaching hours or days, it is necessary to identify media and a sampling protocol that maintains the viability of bacteria throughout the sampling period. It is also desirable to avoid bacteria growth, as the number of bacteria collected should remain consistent throughout the duration of the sampling. A further challenge involves downstream, post-collection analysis, which following traditional impaction, involves incubation of the media and colony counting to estimate of the density of airborne bacteria. The drawbacks associated with the current state of post-collection analyses include (i) poor reproducibility, (ii) the need for multiple culture

media (and associated incubation parameters) when assaying for multiple organisms, and (iii) the inability to perform advanced molecular diagnostics. The development of a protocol to limit these shortcomings would advance our understanding of the composition and dynamics of airborne bacteria communities.

Under the collection conditions defined by traditional impactor use, assessments of microbial densities and community composition are likely inaccurate and unreliable. Therefore, the overall objectives of this study were to:

1) Develop an improved method of sampling bioaerosols over an extended period of time that allows for the representative enumeration and characterization of community structure of airborne bacteria.

2) Test and validate the method in a relevant environment.

CHAPTER 2

Materials and Methods

Methods in brief. To develop a novel sampling method that can assess aerosolized bacteria over extended periods and overcome the drawbacks of conventional impactor sampling, a new type of media was developed. The media was used in conjunction with a protocol that involved attaching two impactors to a low velocity air-pump, which sampled periodically over an extended period. A novel approach for bacteria collection was also developed, as collected bacteria were impacted onto nitrocellulose membranes (0.45 μm pore size) overlaid onto the agar. Once sampling was complete, the membranes were removed from the agar and subjected to microbiological analysis including both culture-dependent (to determine bacteria densities) and culture-independent (to determine bacteria communities) methods.

The novel, periodic sampling method was compared to conventional, continuous sampling simultaneously in a public corridor, and then later applied to two residential indoor environments. Densities of culturable bacteria were determined using plate counts, whereas bacteria community structures were assessed using genetic fingerprinting with denaturing gradient gel electrophoresis (DGGE).

2.1 Selecting appropriate media to maintain bacteria viability during sampling

Extended impactor air sampling times can lead to desiccation of the media in the airstream, as well as the sampled bacteria, limiting the accuracy of the bacteria load estimate. To overcome this limitation, we formulated agar media with a decreased overall agar percentage to effectively increase the medium's moisture content relative to conventional media, which commonly contains 1.5% agar. Since our aim was to collect bacteria and maintain them for an extended time prior to enumeration analysis, we also added non-nutrient buffer to the agar to create an osmotic environment that would best support bacteria viability without promoting growth. Several combinations of buffers and agars were tested, including (i) Amies agar gel with charcoal, (ii) 60 mM phosphate buffered agar (0.4% agar), (iii) 60 mM peptone-phosphate-buffered agar (PPBA) (0.4% and 0.8% agar), and (iv) lake water-buffered agar (0.4% agar) (Figures 2-1, 2-2 and 2-3).

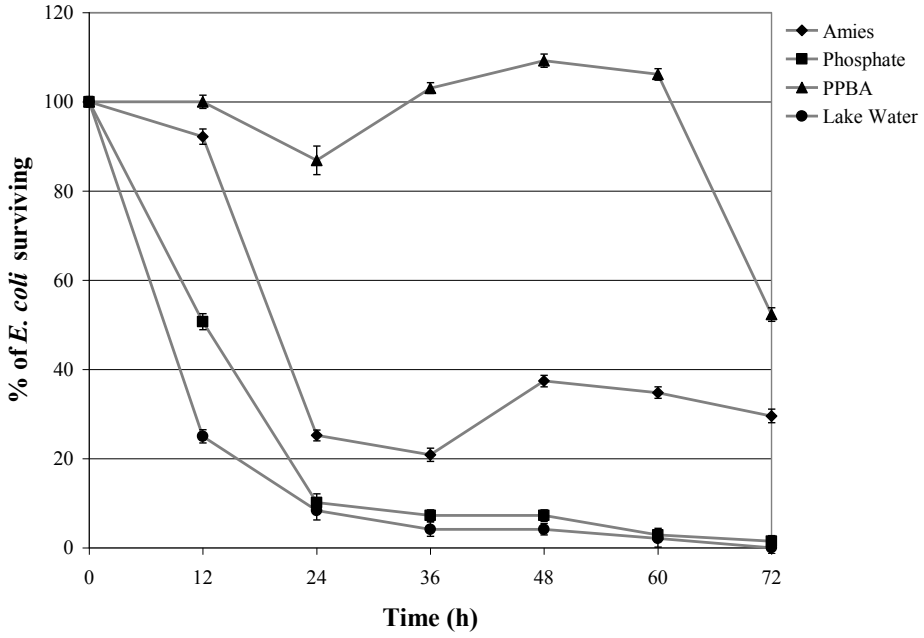

Figure 2-1: Viability of *E. coli* on four different buffered media (0.4% agar) over three days.

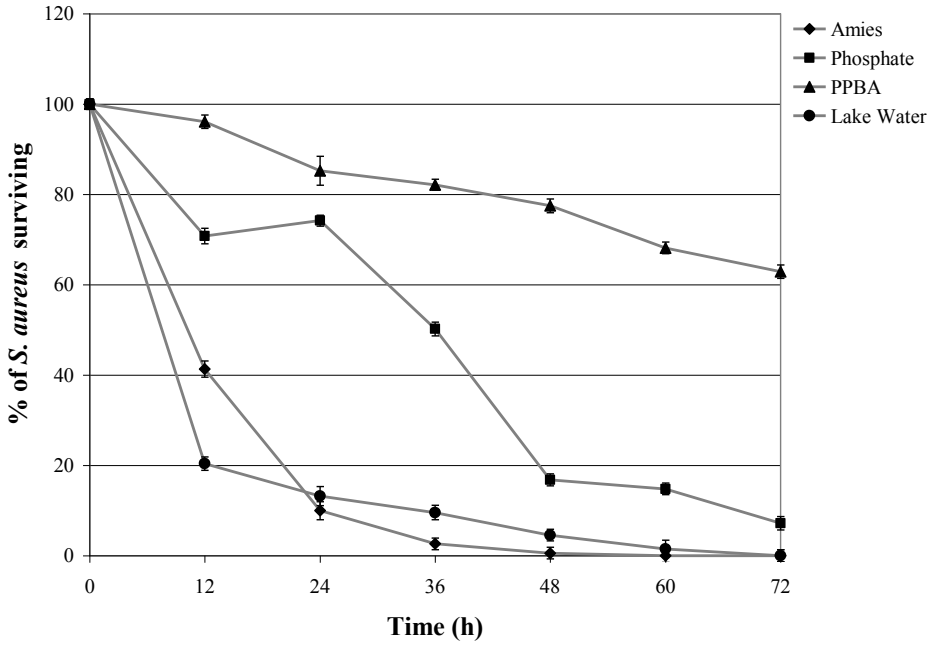

Figure 2-2: Viability of *S. aureus* on four different buffered media (0.4% agar) over three days.

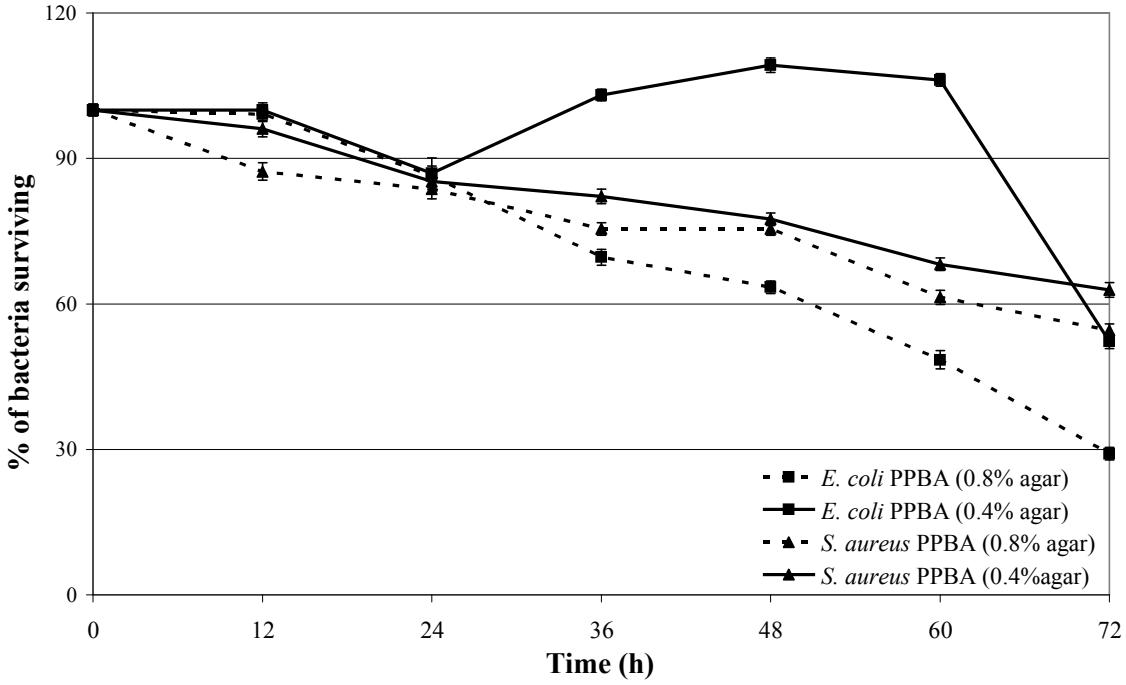

Figure 2-3: Viability of *S. aureus* and *E. coli* on PPBA (0.4 % and 0.8% agar) over three days.

The viability of bacteria on each buffered agar was assessed by filtering approximately 100 *Escherichia coli* JM109 and *Staphylococcus aureus* isolates in phosphate buffer onto a series of sterile nitrocellulose membranes (0.45 μm pore size). The membranes were overlayed onto Petri dishes containing the buffered agars described above and incubated at 25° C. At twelve-hour intervals for up to 72 h, membranes were transferred in triplicate to either RAPID *E. coli* 2 agar (Bio-Rad) or Baird-Parker agar (for *S. aureus*, BD Difco) and incubated at 37° C and 35° C, respectively for 24 h. Colonies were counted and results were expressed as the number of bacteria remaining viable per unit time. The viability experiments suggested that the 60 mM peptone-phosphate buffered agar (PPBA) (0.4% agar) most effectively facilitated the survival of *E. coli* (Figure 2-1) and *S. aureus* (Figure 2-2) over the 72 h incubation period. Additionally, the viability of each bacterium was enhanced on 0.4% agar, as compared to 0.8% agar (Figure 2-3) and was therefore utilized throughout the remainder of the study. However, since the viability of *E. coli* decreased rapidly after 48 h, the air sampling duration was limited to 48 h.

2.2 Determining the impact of sampling frequency on bacteria enumeration and community structure.

To determine how the frequency of sampling impacted the number and community structure of airborne bacteria, air sampling was performed using continuous and periodic sampling methods simultaneously in a public corridor adjacent to the main entrance to a university building. In general, bacteria were collected using two, single-stage, viable cascade impactors (SKC BioStage 200) attached to a low velocity air pump (F&J Model

DF-40L-8) calibrated to provide 28.3 L min^{-1} of air flow, which provided a flow rate of approximately 14 L min^{-1} to each impactor. Each air pump was connected to an electric timer (F&J Model DF-60810E), set to activate the pumps as defined by the sampling method. Three sets of adjacent impactors (SKC Standard Biostage), each mounted on a tripod at a height of 1.5 – 2 m and separated by approximately 1.2 m were located approximately 3 m, 6 m and 8 m from the main corridor entrance. To effectively compare both sampling methods, equivalent air sampling times were used (60 min). However, the traditional sampling was performed for 60 minutes continuously (termed "continuous sampling"), while the novel, "periodic sampling" method was performed for 10 min h^{-1} for 6 h. For periodic sampling, continuous samples were collected in triplicate, while six replicates were collected for each periodic sampling event.

Each impactor contained a 100 mm diameter Petri dish containing PPBA (based on the results of the viability assays described above), overlaid with an 82 mm diameter nitrocellulose membrane (0.45 μm pore size) onto which sampled bacteria were impacted. The use of a membrane to capture impacted bacteria is novel and was utilized in this study to facilitate flexibility of downstream applications (described below). After each sampling event, the Petri dishes were covered and maintained at 4^{o} C until analysis was performed (within 6 hours of collection). Between each sampling event, the impactors were cleaned with 70% alcohol and autoclaved. Sampling pumps were calibrated using a DC-Lite Dry calibrator (Brandt Instruments, Inc.) before and after each sampling session.

To assess how the density of airborne bacteria can fluctuate over time, periodic sampling was performed, as described above, but without a membrane to collect bacteria.

Instead, each impactor was supplied with a Petri dish containing tryptic soy agar, and the media was replaced after every 10 minutes sampling period. All samples were collected in triplicate and stored at 4^{o} C until analysis. Results were expressed as CFUs of bacteria collected m^{-3} throughout the six hour sampling.

2.3 Microbiological analyses

Membranes were removed from the agar with flame-sterilized forceps and cut in half. One half of each membrane was used to estimate the heterotrophic bacteria load in the air samples. Briefly, each half-membrane was placed into a 50 ml sterile Falcon tube containing 25 ml of 60 mM phosphate buffer (pH 7.0) and shaken on a vortex (highest setting) for 20 seconds to disperse the bacteria. One hundred µl of the suspension was inoculated onto tryptic soy agar (TSA), as well as TSA supplemented with triclosan (1.0 µl ml^{-1}) in an effort to ascertain the airborne density of triclosan-resistant bacteria. All TSA enrichments were incubated at 25° C for 24 h and results were expressed as CFUs of bacteria m^{-3} of air.

The remaining half of each membrane was processed for the isolation of DNA using the UltraClean Water DNA Isolation Kit (MO BIO) according to the manufacturer's instructions. DNA was stored at -20° C.

Media used to determine the fluctuation in bacteria densities was incubated at 25^{o} C for 16 h, following which colonies were counted to estimate the CFUs m^{-3} $time^{-1}$.

2.4 PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analyses

To determine the community structure of the sampled airborne bacteria community, 2 µl of each DNA sample was used in a 50 µl PCR reaction to amplify a portion of the V3 region of the 16S rRNA gene (Muzyer *et al.,* 1993). For each set of PCR reactions, DNA extracted from *E.coli* strain JM109 was used as a positive control, whereas the negative control reaction contained sterile, nuclease-free water instead of DNA. A GC-clamp was added to the forward PCR primer to facilitate PCR product separation during DGGE analysis (Myers *et al.*, 1985). The PCR products were visualized using agarose gel electrophoresis and compared to a 100 bp DNA size standard (Promega, USA), to identify the size of the products (approximately 230 bp) and to confirm that the correct product was amplified. PCR reactions were repeated if a reaction generated a negative result.

DGGE analysis of the PCR products was performed using a DCODE Universal Mutation Detection system (Bio-Rad Laboratories, USA) following the method of Sigler and Pasutti (2006) except for the voltage and duration, which were 60 V for 16 h, respectively, to optimize band clarity and separation. All PCR products were processed in 8% acrylamide gels in a denaturing gradient range of 40-60% (a 100% denaturant concentration is defined as 7 M urea and 40 % deionized formamide). Following DGGE analysis, gels were stained for 20 min in 50 ml of a 1:10,000 dilution of GelStar nucleic acid stain (Biowhittaker). The fingerprint images were documented using a Kodak Gel Logic 200 image analysis system. To allow for both intra and inter-gel comparisons, a DGGE marker was loaded such that marker lanes were adjacent to every five sample lanes. The DGGE marker was developed by combining equal volumes of PCR products generated by amplifying the 16S rDNA (Muyzer *et al.*, 1993) of *E. coli* strain DH5α,

Bacillus subtilis, *Staphylococcus aureus, Pseudomonas aeruginosa, Ralstonia pickettii* and four unknown environmental bacteria. DGGE analysis was repeated at least twice to confirm fingerprint reproducibility.

2.5 Data analysis

Bacteria enumerations were assessed using the t-test function in Microsoft Excel (version 2010) to determine if the difference among the average number of airborne bacteria collected using the two different sampling methods was significant.

All fingerprint images were analyzed using GelCompar II software (Applied Maths Version 4.5). The DGGE marker was used as an external reference for the normalization of all fingerprints, whereas bands that were visually present in four or more fingerprints in each gel were used as internal references. Bands were manually identified following the use of multiple exposure settings and magnification of the images. Similarity matrices were calculated using the Dice index (Dice, 1945). Cluster analysis was performed on the similarity matrices by using the unweighted pair group method with arithmetic means algorithm (UPGMA) to produce dendograms that graphically illustrated the similarities among the bacteria communities.

Aside from analyzing the DGGE fingerprints using the GelCompar II software, the bands representing the bacterial communities from the hallway sampling were counted and analyzed with a t-test to determine if the difference in the average number of bands detected using the two sampling methods was significant.

2.6 Applying periodic sampling to characterize indoor air quality

The periodic sampling method was applied, as described above, to two separate indoor residential units located in the city of Oregon (Ohio), each over a 48 h period. The novel PPBA media was used for these sampling events. Samplings in both houses were repeated three times over a period of 6 weeks to determine the bacteria community structure in an indoor environment.

CHAPTER 3

Results

3.1 Corridor sampling event

3.1.1 Bacteria density trends in continuous vs. periodic sampling

Air samplings performed in a public corridor to determine if there was a difference in the number of bacteria sampled using continuous vs. periodic sampling showed that the number of bacteria detected following periodic sampling were significantly higher (average of 82 CFU m^{-3}) than the number collected using continuous sampling (54 CFU m^{-3}) ($p=0.04$) (Figure 3-1).

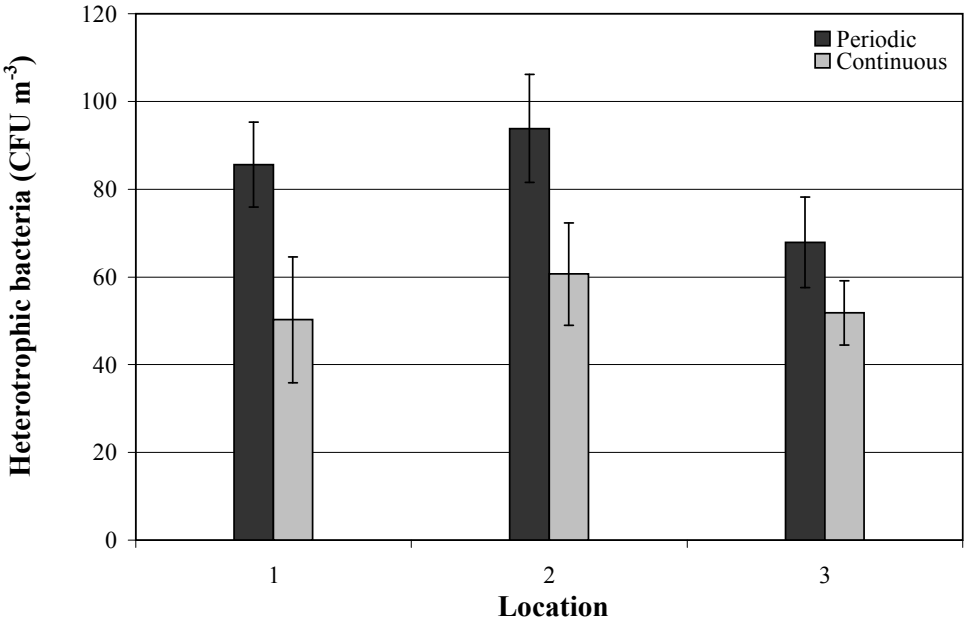

Figure 3-1: Comparison of bacteria density estimates following continuous and periodic sampling in 3 different locations in a corridor

3.1.2 Assessing temporal fluctuation in the number of bacteria sampled

Periodic sampling performed to determine if temporal fluctuations in airborne bacteria density contributed to the differences in the number of sampled bacteria observed between the two methods showed that bacteria densities varied in the three corridor locations (Figure 3-2). In location 1, densities were highest during the fourth hour (80 ± 1 CFU m^{-3} of air) and were lowest during the sixth hour (35 ± 1 CFU m^{-3} of air). In location 2, densities were highest during the first hour (86 ± 1 CFU m^{-3} of air) and were lowest during the third hour (48 ± 1 CFU m^{-3} of air).

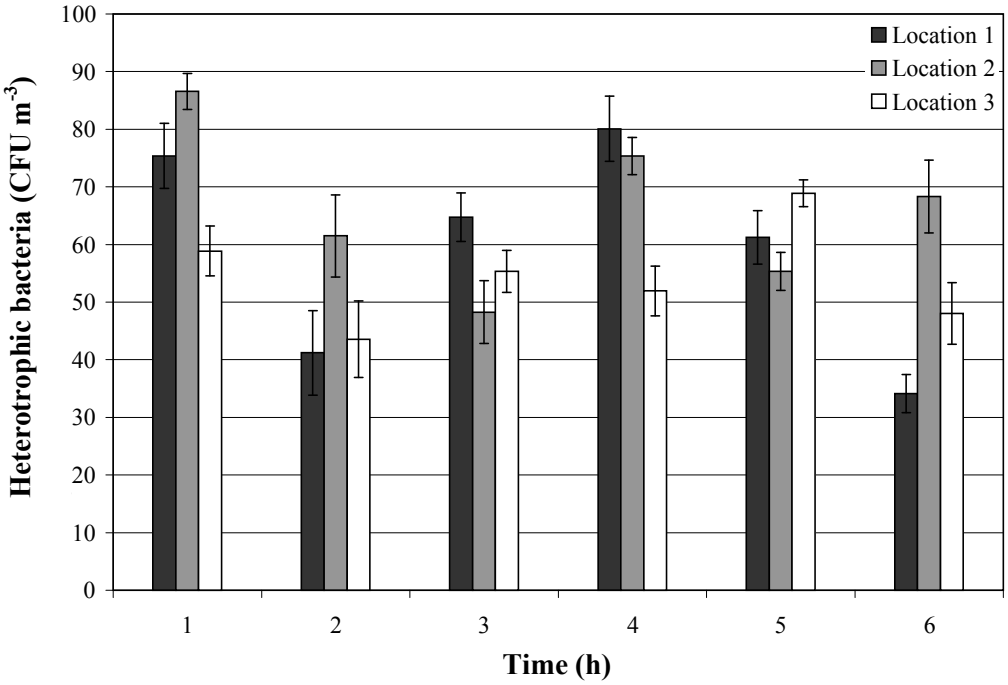

Figure 3-2: Graph showing the fluctuations in bacteria density trends over a 6 h periodic sampling event in three different locations in a corridor

3.1.3 Bacteria community structure

DGGE analysis to determine if differences in the structures of the bacterial communities were detected following periodic and continuous sampling revealed that the two sampling methods differed significantly with regard to the number of bands (a proxy for the number of bacteria communities sampled) visually detected following DGGE analysis ($p=0.01$) (Table 3.1). Specifically, fingerprints of samples collected using periodic sampling contained an average of 30 bands, whereas continuous sampling resulted in fingerprints containing an average of 21 bands. Cluster analysis of the resulting fingerprint similarity matrices revealed that the bacteria communities captured using periodic sampling were dissimilar to those captured using continuous sampling.

Specifically, bacteria communities in the three corridor locations sampled with periodic sampling were 41%, 34% and 42% similar to the communities sampled with continuous samplings (Figure 3-3).

Table 3.1: Number of bands (average) in the DGGE fingerprints resulting from samples collected during the corridor sampling event

Location in corridor	Continuous sampling (60 minutes)	Periodic sampling (10 min h^{-1} over 6h)
1	15	24
2	20	33
3	22	34

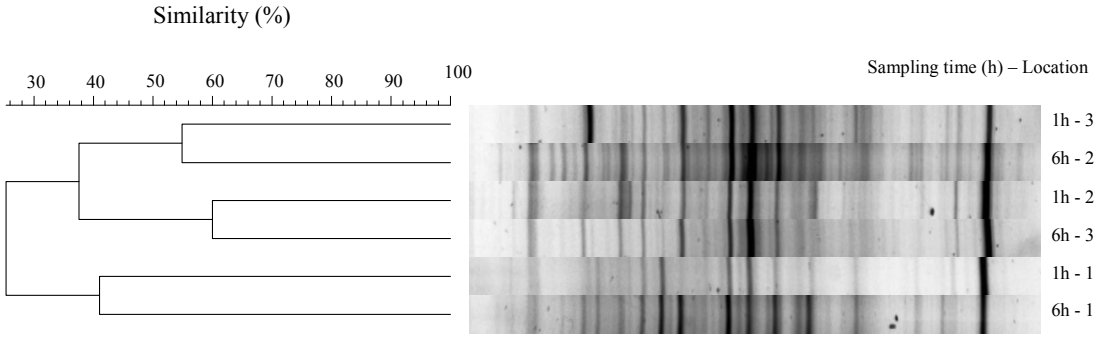

Figure 3-3: Comparison of bacteria community similarity following continuous and periodic sampling, in 3 different locations in a corridor. 1h - 1 represents continuous sampling for 1 h in location 1 in the corridor, whereas 6h - 1 represents periodic sampling for 10 min h^{-1} for 6 h in location 1 in the corridor

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3.2 House sampling

3.2.1 Bacteria densities in houses

The densities of bacteria collected from both houses varied significantly throughout the three sampling events, ranging from an average of $(1.9 \pm 0.1)*10^7$ to $(5 \pm 0.1)*10^7$ CFU m^{-3} in House 1, and $(7.3 \pm 0.1)*10^7$ to $(9 \pm 0.1)*10^7$ CFU m^{-3} in House 2 (Figures 3-4, 3-5 respectively). Additionally, bacteria were heterogeneously distributed, as densities of bacteria collected with impactors placed approximately 1.2 m apart and sampling simultaneously often varied significantly. For example, during the third sampling event in House 1, densities of bacteria collected averaged $(6 \pm 0.4)*10^7$ CFU m^{-3} (impactor 1) and $(4 \pm 0.3)*10^7$ CFU m^{-3} (impactor 2). Likewise, during the first sampling event in House 2, densities averaged $(6 \pm 0.1)*10^7$ CFU m^{-3} (impactor 1) to $(8 \pm 0.3)*10^7$ CFU m^{-3} (impactor 2) (Figures 3-4, 3-5, respectively).

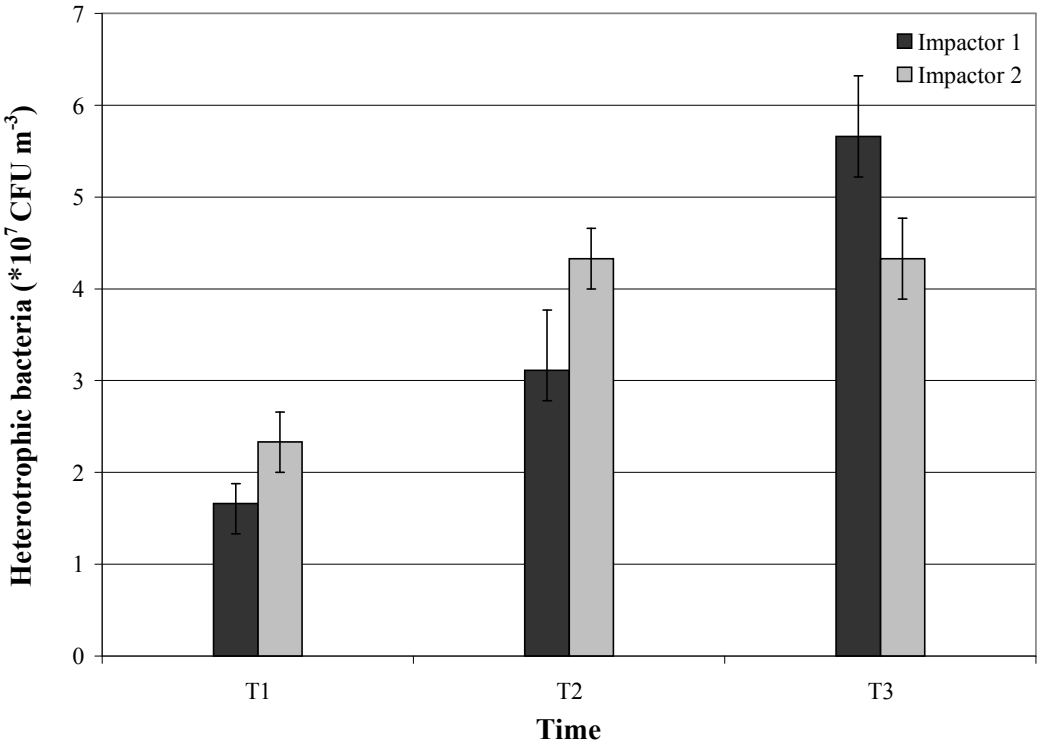

Figure 3-4: Bacterial Bacteria density on tryptic soy agar, from periodic sampling (10 min h^{-1} over a two day period) in House 1

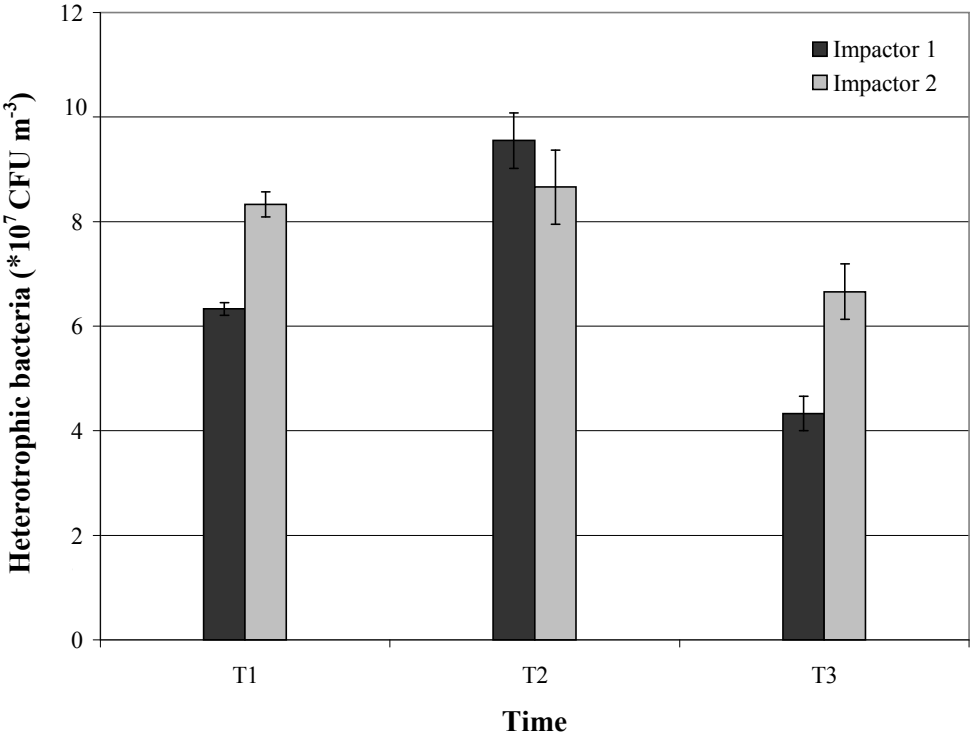

Figure 3-5: Bacteria density on tryptic soy agar, from periodic sampling (10 min h^{-1} over a two day period) in House 2

Analysis to determine densities of airborne, triclosan-resistant bacteria showed that triclosan-resistant bacteria were observed in both houses during each of the three sampling events, comprising approximately 50% of the total bacteria detected. The number of resistant bacteria ranged from an average of $(0.5 \pm 0.04)*10^7$ to $(3 \pm 0.05)*10^7$ CFU m^{-3} (House 1) and $(2 \pm 0.1)*10^7$ to $(4 \pm 0.2)*10^7$ CFU m^{-3} (House 2) (Figures 3-6, 3-7 respectively). Heterogeneity was also observed in the densities of triclosan-resistant bacteria collected from adjacent, simultaneously sampling impactors. For example, during the second sampling event in House 1, densities of bacteria collected averaged $(3.1 \pm 0.05)*10^7$ CFU m^{-3} (impactor 1) and $(2.3 \pm 0.1)*10^7$ CFU m^{-3} (impactor 2). Likewise, during the second sampling event in House 2, densities averaged (1.6 ±

0.03)*10^7 CFU m^{-3} (impactor 1) to (2.3 ± 0.7)*10^7 CFU m^{-3} (impactor 2) (Figures 3-6, 3-7 respectively).

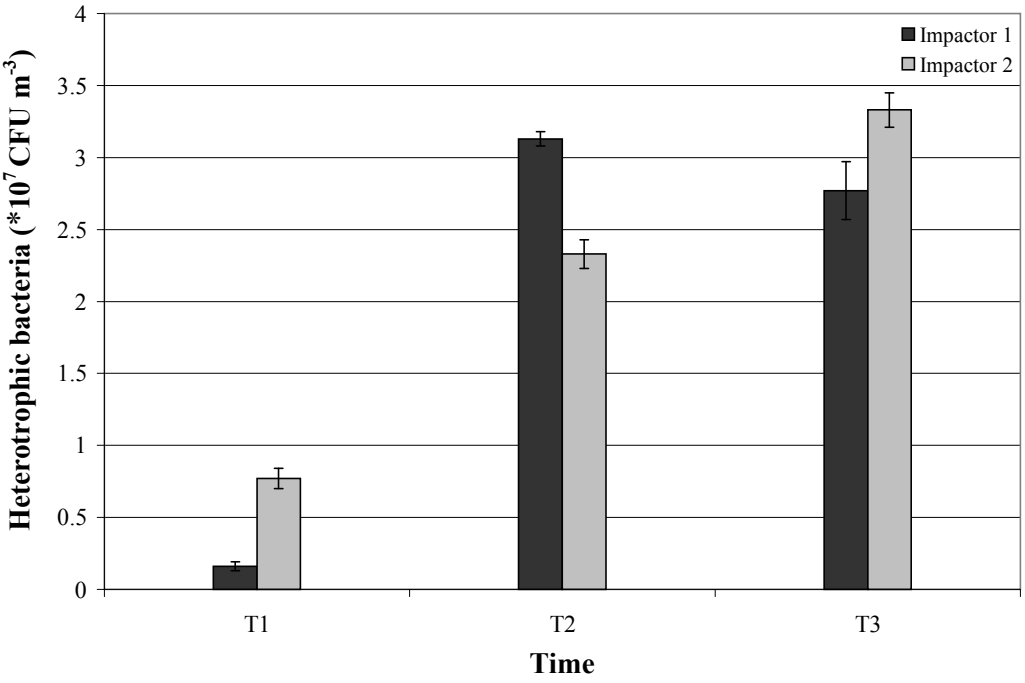

Figure 3-6: Bacteria density on tryptic soy agar with triclosan (1.0 µl ml^{-1}), from periodic sampling (10 min h^{-1} over a two day period) in House 1

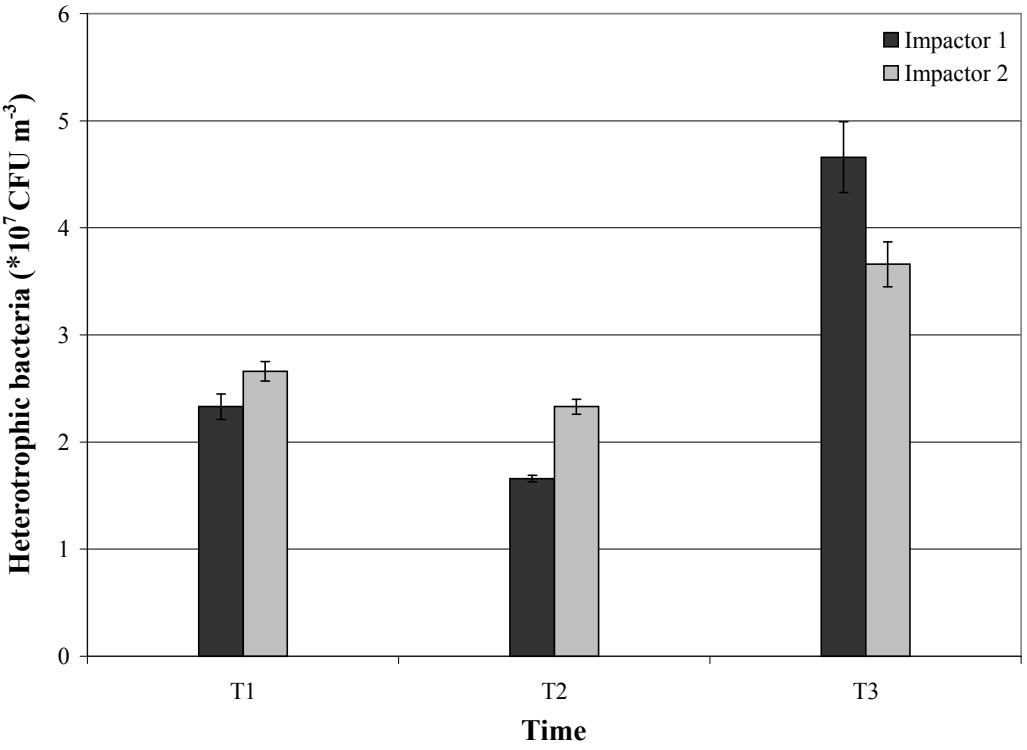

Figure 3-7: Bacterial density on tryptic soy agar with triclosan (1.0 µl ml^{-1}), from periodic sampling (10 min h^{-1} over a two day period) in House 2.

3.2.2 Bacteria community structure

Community structure analysis using DGGE was performed on the bacteria communities collected in both houses to determine the temporal dynamics in community structure during the six sampling events. DGGE analyses revealed that community composition varied significantly between sampling times in both houses. In House 1, the communities collected during sampling event 1 were 41% and 31% similar to those from sampling events 2 and 3, respectively, whereas the communities collected during sampling event 2 were 33% similar to those collected during event 3 (Figure 3-8).

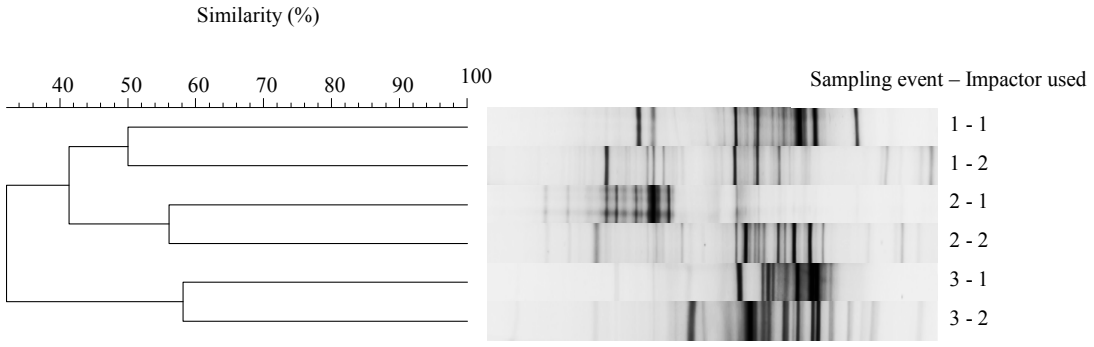

Figure 3-8: Comparison of bacteria community following periodic sampling in House 1 over three different time periods (1, 2 and 3). 1 – 1 represents bacteria communities collected by impactor 1 during sampling event 1, whereas 1 – 2 represents the bacteria communities collected via impactor 2 during sampling event 1.

In House 2, the communities collected during sampling event 1 were 34% and 19% similar to those from sampling events 2 and 3, respectively, whereas the communities collected during sampling event 2 were 27% similar to those collected during event 3 (Figure 3-9).

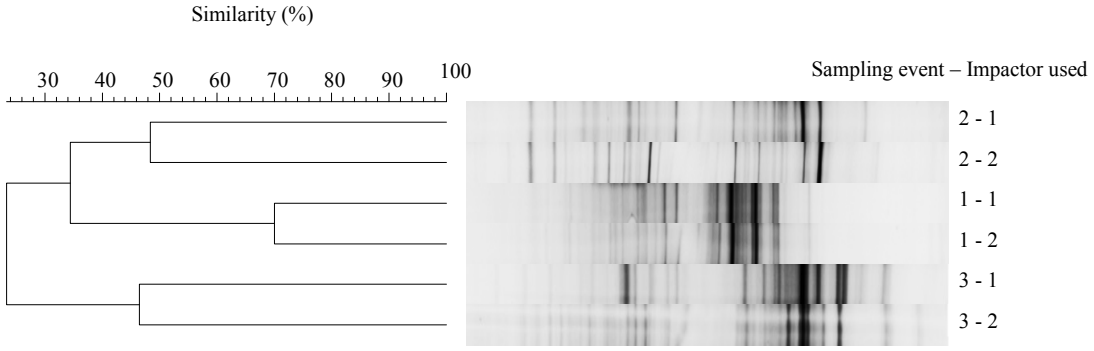

Figure 3-9: Comparison of bacteria community following periodic sampling in House 2 over three different time periods (1, 2 and 3). 1 – 1 represents bacteria communities collected by impactor 1 during sampling event 1, whereas 1 – 2 represents the bacteria communities collected via impactor 2 during sampling event 1.

Comparison of the bacteria communities collected from adjacent impactors that were sampling simultaneously revealed significant heterogeneity between samplers in all sampling events. For example, in House 1, the communities collected during sampling event 1 from impactor 1 were 50% similar to those from impactor 2. The communities collected during sampling event 2 from impactor 1 were 56% similar to those from impactor 2. The communities collected during sampling event 3 from impactor 1 were 58% similar to those from impactor 2. Similarly in House 2, the communities collected during sampling event 1 from impactor 1 were 70% similar to those from impactor 2. The communities collected during sampling event 2 from impactor 1 were 48% similar to those from impactor 2 and communities collected during sampling event 3 from impactor 1 were 46% similar to those from impactor 2.

CHAPTER 4

Discussion

Despite their significant impact to public health, airborne microbes remain poorly characterized in the atmosphere and in indoor environments (Peccia *et al.*, 2006). One factor contributing to our lack of understanding is the low abundance of airborne bacteria, which is, on average, 100 cells m^{-3} (Tong *et al.*, 1999). In contrast, for example, total bacteria abundance in soils can exceed 10^8 CFUs g^{-1} (Turco *et al.*, 1995; Hazen *et al.*, 1991), whereas fecal bacteria densities, indicators of water quality, can exceed 10^{11} CFUs m^{-3} in impaired systems (Niemi *et al.,* 1991). Furthermore, some sources might release bioaerosols of public health importance as "concentration bursts", that can result in up to 22,000 CFU m^{-3} in indoor environments, in irregular frequency and lasting only seconds (Burge, 1995; Macher *et al.*, 1995, Lai *et al.*, 2003). Effective detection and characterization of the airborne bacteria community is therefore dependent on (i) sampling an adequate volume of air to detect microbes distributed heterogeneously and in low density, and (ii) downstream microbiological and molecular analyses (Fahlgren *et al.*, 2011).

Bioaerosol sampling can be performed using impaction devices, which offer the advantage of directly collecting viable biological agents. Impaction involves the

collection of airborne bacteria by drawing an airstream through a manifold and onto agar media, where the collected bacteria can be grown and enumerated using traditional plate counting analysis. Since conventional, one time bioaerosol sampling is usually conducted over short-term periods (15 minutes) (Douwes *et al.*, 2003), it is mostly non-representative, especially considering that some sources might release bioaerosols, that could pose as potential public health hazards, in irregular frequency and lasting only seconds (Burge, 1995; Macher *et al.*, 1995). However, extended sampling times can result in desiccation of the agar media, resulting in the death of collected bacteria (Stetzenbach *et al.*, 2004) and a subsequent underestimation of bacteria load. Because of the low densities and heterogeneous distribution of airborne bacteria, conventional sampling times might not allow for the collection of a representative volume of air appropriate for microbiological analyses. Therefore, it follows that, air sampled periodically throughout extended periods might allow for improved representation of the microbial community and an understanding of the relationship between the density of biological agents and health effects (Harrison *et al.*, 1992; Nelson *et al.*, 1995). To the best of our knowledge, no method has been devised that facilitates the sampling and maintenance of live bioaerosols for periods extending beyond several minutes without the necessity of constant/periodic monitoring. To extend sampling times, while maintaining viable bacteria, impaction media must be developed that can limit the desiccation of impacted bacteria in the airstream, as bacteria collected early in the sampling campaign are subjected to continued airflow during the subsequent sampling period. The media used in our periodic sampling method (PPBA with 0.4% agar) effectively maintained approximately 100% viability in *E. coli* and 80% viability in *S. aureus*, over a two day

period (Figures 2-1 & 2-2). Preliminary assays showed that media containing conventional agar content (1.5%) desiccated rapidly over the 2 day period due to its relatively low moisture content and was unable to maintain bacteria viability. We attributed the enhanced survival in PPBA to elevated moisture content, which limited the desiccation of collected bacteria during long-term sampling. Subsequently the improved media likely allowed for the collection and maintenance of a more representative community of aerosolized bacteria, as compared to traditional media, by maintaining the viability of bacteria over a longer duration.

Unlike continuous sampling, which took place throughout continuously for 60 minutes, periodic sampling involved ten minutes of sampling each hour over a six-hour duration and could resolve the fluctuations in bacterial densities that occurred over time. By facilitating the characterization of short-term fluctuations in bacteria density, periodic sampling improved upon continuous sampling, during which it is not possible to capture the bacterial populations on a highly resolved, temporal scale. Although not quantified, the movement and changes in density of people in the corridor, along with the opening/closing of classroom and building entrance doors throughout the sampling period likely influenced the dynamics of the bacteria populations. Our findings were consistent with those of Brandl *et al.*, (2008) who observed the occurrence of distinct and reproducible short-term dynamics (on a time scale of minutes) of total particles and bioaerosols, in an university hallway, related to periods of anthropogenic activity (presence/absence of people). The community dynamics were also illustrated by genetic fingerprints that showed that the number of bacteria communities was significantly

higher in the samples collected during periodic sampling, than from samples collected using traditional sampling ($p=0.01$). Therefore, while it has been established that increased sampling times can improve sampling representation, decrease variability between samples, and more accurately assess exposure risk, as compared to traditional short term sampling events (Lee *et al*., 2006; Lin *et al.*, 1999; Pasanen, 2001), we maintain that sampling periodically can also enhance the representative characterization of the airborne microbial community.

Analyses of samples from traditional bioaerosol samplers depend mainly on culture dependent methods (Chen *et al.*, 2004). Furthermore, non-viable organisms, or those that are not culturable under selected culture conditions, can sometimes pose the greatest public health risk (Byrd *et al.*, 1991; Hussong *et al.,* 1987 and Samet *et al.,* 2004). While culture-based analysis enables the detection and quantification of some bacteria of public health relevance (Ellis *et al.*, 2003), less than 1% of the total airborne microbes are detected (Peccia *et al.*, 2006). Therefore, bacteria densities enumerated by conventional culturing alone cannot be used to comprehensively investigate airborne microbial communities. Several recent studies have described culture-independent, DNA-based approaches to better characterize the diverse aerosolized bacterial communities (Kelley *et al.*, 2004; Pakarinen *et al.,* 2008; Pitkäranta *et al.,* 2008; Rintala *et al.,* 2008 and Täubel *et al.,* 2009). In the current study, we have demonstrated for the first time the use of DGGE analysis as a means to characterize airborne microbial communities. DGGE analysis is used to study complex bacteria communities and has been shown to be more effective than culture-dependent analyses for characterizing bacteria community structures

(Muyzer *et al.,* 1998; Temmerman *et al.*, 2003). DGGE analyses of the corridor communities (Figure 2-3) revealed that periodic sampling detected a significantly greater number of bacteria communities (as evidenced by the number of DGGE bands) than the continuous sampling. Further analyses revealed variability in communities collected during different sampling periods, including significant variability between the community structures of bacteria collected from adjacent impactors sampling simultaneously (Figures 3-8 & 3-9). Our results supported the findings of others who reported that even when samples are collected from a single, confined location, the airborne microbial community is dynamic and can differ significantly spatially and temporally across coarse and fine scales (Bovallius *et al.*, 1978; Lighthart, 1997 & Lighthart *et al.*, 1994; Fierer *et al.*, 2008).

The most innovative aspect of the periodic sampling method was the impaction of aerosolized bacteria on to a nitrocellulose membrane (0.45 μm pore size) overlaid onto the PPBA (0.4% agar) media, as opposed to traditional, direct impaction onto the agar surface. The use of a membrane to capture airborne bacteria for community structure analyses has not been previously attempted. Since DNA extraction from bacteria collected directly onto the agar is not possible without first introducing biases associated with growth, our community analyses would not have been possible without utilizing an impaction surface from which DNA could be isolated. The flexibility of using a membrane for impacting airborne microorganisms allowed for (i) accurate enumeration by using culture-dependent techniques to determine bacteria densities, and (ii) community analysis, by using culture-independent methods (DNA extraction, PCR analysis, DGGE fingerprinting) to analyze the bacteria communities. While our results

indicated the flexibility of using an impaction surface, the collection efficiency of the membrane should be investigated and requires further study.

Microbes are distributed heterogeneously throughout the atmosphere (Fahlgren *et al.,* 2011) and periodic samplings in two residential indoor environments revealed that densities of bacteria collected from adjacent samplers were significantly different (Figures 3-4 and 3-5), as were densities observed in the two houses during three sampling events. (Figures 3-4 and 3-5) These findings showed that indoor airborne bacteria densities, distribution, and temporal and spatial fluctuations can occur on small time scales (within minutes). This dynamic depends on several activities, including the movement of people, presence of pets, and cooking and, cleaning practices, among others (Abt *et al.*, 2000; Luoma *et al.*, 2001 and Morawska *et al.*, 2003). Our findings were also consistent with those who reported that even when samples are collected from one location, the types of microbes present in that atmosphere can change significantly across a given day, month, or year (Bovallius *et al.*, 1978, Lighthart, 1997 & Lighthart *et al.*, 1994).

The emergence of infections caused by antibiotic-resistant pathogens is a growing problem and has now become a major health issue (Bonomo *et al.*, 1999). However, little data exist on the concentration of bacteria in indoor air in comparison to other airborne contaminants (Gandara *et al.,* 2006). In the current study, triclosan-resistant bacteria were not only present in the residences sampled over a two-day period, on average, they comprised approximately 50% of the total airborne bacteria community (Figures 3-6 and

3-7). This proportion is much greater than what was found by Patrikis *et al.,* (2009) who reported that the highest antibiotic resistant in indoor airborne bacteria was found for Ampicillin varying from 1.4 – 7.8% of the total indoor airborne bacteria population. Gandara *et al*., (2006) showed that densities of aerosolized, multidrug-resistant *S. aureus* were higher in the indoor environments of residences than outside the homes. Human activities within houses, such as movement of curtains, bed sheets and dry fabrics increases the concentrations of aerosolized bacteria within homes, which may also suggest that such activities also elevate the levels of antibiotic resistant bacteria within homes (Gandara *et al*., 2006).

Overall, our study was focused on developing a novel method to successfully sample viable bioaerosols for an extended period. Key to meeting this objective was overcoming the limitations associated with sampling with conventional impactors for relatively short term periods. Unlike samples collected from conventional impactors, samples collected using periodic sampling can be analyzed using not only traditional, culture based analyses but also culture-dependent and independent methods. This allows for the characterization and representation of more bacterial communities in the atmosphere, which could be of great importance to public health.

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