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Evaluation of quantitative polymerase chain reaction for measuring the concentration of total bacteria in environmental air samples

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EVALUATION OF QUANTITATIVE POLYMERASE CHAIN REACTION FOR MEASURING THE CONCENTRATION OF TOTAL BACTERIA IN ENVIRONMENTAL AIR SAMPLES

by

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ABSTRACT

Evaluation of Quantitative Polymerase Chain Reaction for Measuring the Concentration of Total Bacteria in Environmental Air Samples

by

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The air quality of both indoor and outdoor environments is a primary human health concern, particularly for individuals that have asthma, respiratory ailments and immune disorders. Airborne microorganisms have been shown to cause a variety of diseases, allergic reactions, and irritations. Universal quantitative polymerase chain reaction (QPCR) was compared to the traditional methods of culture analysis and microscopy to determine if it was an effective method to quantitate total bacterial counts in environmental air samples. A composite standard curve was developed using four bacterial species and applied to laboratory cultures and environmental air samples. Two hypotheses were tested, (i) to determine if universal QPCR was a more sensitive method to analyze environmental air samples and (ii) if universal QPCR can provide a more accurate measurement of airborne bacteria than culture analysis or microscopy. A total of 22 air samples were collected with an SKC BioSampler® and were analyzed by culture, microscopy and universal QPCR. Results showed microscopy being able to determine higher bacterial concentrations as compared to universal QPCR. However, microscopy

may over-estimate those concentrations. It was concluded that universal QPCR was a more sensitive method than culture or microscopy when comparing the lower detection limit (LDL) of each method. Universal QPCR was determined to be a relatively accurate method to assess airborne microbial populations compared to microscopy. Culture analysis cannot determine total bacterial concentrations therefore it was not included when assessing accuracy of universal QPCR. It was also noticed that universal QPCR is not truly universal. Specificity testing revealed that some species did not amplify with universal QPCR. Further research needs be conducted to strengthen the method of universal QPCR.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER 1

INTRODUCTION

The air quality of both indoor and outdoor environments is a primary human health concern, particularly for individuals that have asthma, respiratory ailments and immune disorders (Cox and Wathes, 1995; Stetzenbach *et al.*, 2004). As a result poor air quality has become an increasing public health concern (Douwes *et al.*, 2003). Airborne microorganisms have been shown to cause a variety of diseases, allergic reactions, and irritations (Stetzenbach, 2007). Some microorganisms produce spores that are hardy and persist in the environment for years; some can cause infection long after the initial exposure has occurred and can also cause hypersensitivity diseases (Burge, 1990). Anthropogenic influences and natural environmental variations may alter atmospheric microbial composition (Brodie *et al.*, 2007). Continuous construction of commercial, industrial and private buildings and the dry drought-stricken environment produce outdoor air that is often inundated with particulate that may contain high levels of microorganisms. Therefore, the Las Vegas Valley experiences periods of poor air quality throughout the year (Clark County Department of Air Quality and Environmental Management, 2007). Indoor environments are also at risk of poor air quality (Stetzenbach, 2007; Hirvonen *et al.*, 2005; Douwes *et al.*, 2003). Faulty construction, broken pipes and leaking air conditioning systems all have an impact in creating conditions that enhance the growth of bacteria and fungi indoors. These problems lead to

water damage and resulting microbial growth on building materials and furnishings that deteriorate the materials and may impact the health of occupants (Stetzenbach, 2007). For indoor environments, the ability to detect and identify airborne microorganisms can help determine bio-contamination and the presence of pathogens that can affect healthy individuals.

Airborne microorganisms are measured by air sampling. Commonly used methods to sample air include impaction, liquid impingement, and filtration (Grinshpun *et al.*, 2007). The samples that are collected by these methods can then be analyzed. The advantage of using an impinger is that airborne particles are deposited into a liquid collection buffer and the liquid can be analyzed by several means including culture, microscopy, molecular methods, immunoassays and biochemical assays (Cruz and Buttner, 2007; Stetzenbach *et al.*, 2004). Culture is commonly used to characterize and quantitate airborne microorganisms. Culture analysis involves inoculation of nutrient media and enumeration of microorganisms that grow and produce visible colonies (Cruz and Buttner, 2007). The disadvantage of culture-based analysis is that only a fraction of viable organisms are capable of growth on laboratory media. Culture-based techniques identify less than 1% of all microbial populations in an environmental sample (Farris and Olson, 2007; Amann *et al.*, 1995; Borneman *et al.*, 1996). Culture also can take days to weeks to produce results (Cruz and Buttner, 2007).

Microscopy-based analysis involves enumerating microorganisms using a microscope. Enhanced microscopy can be obtained using a fluorescent stain. The disadvantage of microscopy is that it is labor intensive, expertise is needed for identification, and it has poor sensitivity (Grinshpun *et al.*, 2007). Microscopic analysis

of bacteria provides data on total cell concentrations, but cannot assess viability or discriminate between genera or species. In addition, the time needed to complete both culture and microscopic methods can delay decisions as to the contamination of the sampled area and potential effects on the health of building occupants.

Advances in molecular biology have provided alternatives to traditional analysis methods. Molecular methods such as polymerase chain reaction (PCR) have had tremendous success in advancing the analysis of environmental samples (McDevitt *et al.*, 2004), and molecular methods have been shown to detect low numbers of organisms (Tsai and Olson, 1992).

PCR is a procedure used to rapidly amplify specific DNA sequences (Saiki *et al.*, 1985). This technique has been used successfully to enhance the detection of microorganisms in a variety of matrices (Cruz and Buttner, 2007). Application of the PCR technique to environmental sampling provides an alternative to culture or microscopic enumeration. The use of PCR is particularly suited for detection of microorganisms that are difficult to culture, grow very slowly, or have never been cultured *in vitro* (Cruz and Buttner, 2007). Advantages of PCR over traditional methods are that it is rapid, results are obtained quickly, and a small amount of sample is needed. Culture may take days to weeks to grow before results are obtained (Cruz and Buttner, 2007). PCR can be used for direct detection of a single organism or as a multiplex assay amplifying several target organisms. However, there are limitations of the method, such as the inability to differentiate between viable and non-viable cells. In addition, the

presence of PCR inhibitors may result in false negatives and reduce sensitivity (Cruz and Buttner, 2007).

Quantitative PCR (QPCR) is a recently developed technology that allows sensitive, specific detection and enumeration of target microorganisms. It is a real-time assay that measures product accumulation with fluorogenic probes (Cruz and Buttner, 2007). The assay requires binding of a forward primer and reverse primer to a specific location on the target DNA. A TaqMan® probe, which contains a fluorescent dye, binds between the primers. When DNA is being synthesized the probe is cleaved producing a fluorescent signal. The detected fluorescent signal is reported as the cycle threshold (C_T) value. The C_T value is the cycle where fluorescence is first detected crossing the threshold. It is inversely proportional to the concentration of the sample. The amount of fluorescence measured is used to determine the amount of DNA in a sample. However, to use PCR or QPCR, the DNA sequence of the target organism(s) must be known and specific, and complimentary DNA primers and probes must be developed. An alternative to PCR and QPCR for detection of bacteria is universal PCR.

Universal QPCR is a method that amplifies organisms using a primer and probe set that targets the 16S rDNA region which is highly conserved in all bacteria. Universal QPCR has been developed to study microbial diversity in environmental and clinical samples (Blackwood *et al.*, 2005; Fierer *et al.*, 2005; Nadkarni *et al.*, 2002; Suzuki *et al.*, 2000). Most studies using universal QPCR have focused on clinical samples and environmental soil and aquatic samples (Nadkarni *et al.*, 2002; Horz *et al.*, 2005; Suzuki *et al.*, 2000; Takai and Horikoshi, 2000). Previous studies have shown that universal QPCR is not truly universal in that some organisms are not detected and there is

variability in amplification between species (Buttner and Cruz, 2006). To date, it is not known why certain organisms do not amplify with universal bacterial PCR. A published primer and probe set (Nadkarni *et al.*, 2002) was previously tested to determine the ability to amplify the DNA of representative bacteria from every bacterial phylum (Buttner and Cruz, 2006). The Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA) was used to perform universal QPCR analysis. Results showed that 6 of the 39 representative bacteria did not amplify when using optimized universal primers and probe. In addition, bacteria did not amplify with the same efficiency when using the same amount of DNA template. However, the universal primers and probe did not cross-react with non-bacterial organisms.

The purpose of this study was to evaluate the utility of universal QPCR to detect and quantify total airborne bacteria in environmental air samples as compared to traditional methods of culture and microscopy. To effectively determine if universal QPCR is a suitable tool, two hypotheses were tested in this study. The first hypothesis was: universal QPCR provides a more sensitive measurement of airborne bacteria than culture or total direct microscopic enumeration. This hypothesis was tested by determining the lower detection limit (LDL) of the universal QPCR assay and comparing it to the LDL of the two traditional methods, culture and microscopy. The second hypothesis was: universal QPCR provides a more accurate measurement of airborne bacteria than culture or microscopy. This hypothesis was tested by preparing universal QPCR standards of known concentration with four bacterial species and comparing the three methods, culture, microscopy (acridine orange direct counts, AODC), and universal QPCR. Statistical analysis of the results was conducted by analysis of variance

(ANOVA), and the accuracy of microscopy and universal QPCR were compared to the reference method of electronic particle enumeration. The results of this study are expected to provide a more accurate and sensitive method for determining airborne bacterial concentrations in environmental air samples.

CHAPTER 2

MATERIALS AND METHODS

Experimental Design

To evaluate the utility of universal QPCR for detection and quantification of total airborne bacteria, universal QPCR was compared with two traditional methods, culture analysis and microscopy, in laboratory and field experiments. Field air samples were collected (n=22) using a volumetric air sampler at various locations in the Las Vegas Valley. Culture analysis, microscopic analysis, and universal QPCR were performed on all environmental samples.

Laboratory experiments were conducted using *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus cereus*. Each organism was cultured in nutrient broth, grown to log phase and then electronically enumerated. Mixed cultures containing equal concentrations of the three test bacteria were prepared at three concentrations, 3.0×10^7 cells/ml, 3.0×10^5 cells/ml and 3.0×10^3 cells/ml. Aliquots of each culture were reserved from each concentration for DNA extraction and universal QPCR to determine if there was variability in amplification among the three species that could influence universal QPCR amplification values.

The accuracy of universal QPCR was tested by preparing QPCR standards of known concentration with four organisms, *Shewanella oneidensis*, *Pseudomonas aeruginosa*, *Cellulomonas fimi*, and *Bacillus atrophaeus*. These standards were analyzed by:

microscopy and universal QPCR, and the results were compared to the reference method, electronic enumeration, to determine the accuracy of each assay.

The sensitivity of the universal QPCR assay was determined from serial dilutions and amplification of universal QPCR standards to determine the lower detection limit (LDL) of the assay. The LDL of the universal QPCR standards for each organism was compared to the known LDL of culture analysis and microscopic enumeration.

Test Organisms and Culture Media

All test organisms were obtained from the American Type Culture Collection (ATCC; Manassas, VA) with the exception of *Bacillus atrophaeus* (U.S. Army Dugway Proving Ground, Dugway, UT). *Shewanella oneidensis* ATCC 700550D, *Pseudomonas aeruginosa* ATCC 15442, *Cellulomonas fimi* ATCC 484, and *Bacillus atrophaeus* were used in the preparation of universal QPCR standards. *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 15597, and *Bacillus cereus* ATCC 14579D were used in laboratory experiments. All organisms were cultured on Tryptic Soy Broth (TSB, pH 7.0, Difco, Sparks, MD) or Tryptic Soy Agar amended with cycloheximide, final concentration 100μg/ml (TSAC; pH 7.0, Difco) and incubated at 28°C for 24 to 48 hours.

Air Sampling

Field sampling was conducted using an SKC BioSampler® (SKC Inc., Eighty Four, PA), a liquid impingement sampler used to collect airborne microorganisms (Grinshpun *et al.*, 2007). Twenty ml of sterile 0.01 M potassium phosphate buffer (PB, pH 7.0) was added to a sterile BioSampler®, and was operated for 10 min at a flow rate of 12.5

liters/min. An aliquot was cultured as indicated below and the remaining sample was preserved at -70°C for microscopic and universal QPCR analyses. Different environments in the Las Vegas Valley were selected at random for sampling airborne microorganisms (Table 1).

Table 1. Locations where environmental air samples were collected and the number of samples collected at each location.

Location	Number of samples (n=22)
Cattle yard	2
Desert garden	1
Indoor arena	2
Urban river	6
Kennel (indoor)	4
Barn	5
Field*	2

*Field contained temporary stalls for horses

Preparation of Mixed Cultures

The test organisms *E. coli*, *S. aureus*, and *B. cereus* were cultured in TSB as indicated above. Cells were grown to log phase and then electronically enumerated as indicated below. Each test organism was diluted with PB to three different concentrations; 1.0 x 10^7 cells/ml, 1.0 x 10^5 cells/ml, and 1.0 x 10^3 cells/ml. The test organisms were combined in equal concentrations for a final concentration in each sample of 3.0 x 10^7 cells/ml, 3.0 x 10^5 cells/ml, and 3.0 x 10^3 cells/ml. Pure cultures of each organism were also prepared at the same three concentrations. Both the mixed cultures and pure cultures were tested by universal QPCR to determine if there was any effect on the assay.

Culture Analysis

Culture analysis was performed by plating of the SKC collection buffer. Duplicate 1 ml samples were processed by filtration through a 47-mm-diameter, 0.45-μm-pore-size mixed cellulose-ester membrane (Pall Corp, Ann Arbor, MI) and plating onto TSAC. In addition, samples were serially diluted in PB prior to inoculation onto TSAC. After incubation, the number of colony forming units (CFU) per plate were enumerated; counts from replicate plates were averaged. The number of CFU/ml and the number of CFU/m^3 of air sampled were calculated. The number of CFU/ml were determined by the 1 ml volume filtered and the CFU/plate. The number of CFU/m^3 was determined using the number of CFU/ml, the sample volume, the sampler flow rate and sampling time.

Microscopic Analysis

Microscopic analysis was performed on organisms used for universal QPCR standards and in laboratory experiments by staining samples with 0.1% Acridine Orange with 2% formaldehyde (Hobbie *et al.*, 1977). A 1.8 ml sample aliquot was stained with 200 μl of Acridine Orange (final concentration, 0.01%, Sigma, St. Louis, MO) and incubated at room temperature for 5 min. The stained sample was filtered through a black 25-mm-diameter, 0.2-μm-pore-size nucleopore polycarbonate membrane (Whatman, Florham Park, NJ) and rinsed with PB. The filter was applied to a microscope slide and a drop of immersion oil was placed on the membrane followed by a cover slip. The number of cells was enumerated using epifluorescense microscopy with an oil immersion 100X objective. Twenty fields were counted for each sample using a 0.08mm by 0.08mm ocular grid.

Electronic Enumeration

Organisms that were used in laboratory experiments and for making universal QPCR standards were enumerated electronically. Cultures of *S. oneidensis*, *P. aeruginosa*, *C. fimi*, *B. atrophaeus*, *S. aureus*, *E. coli*, and *B. cereus* were grown on TSAC from freezer stocks and incubated at 28°C for 24 to 48 h. An overnight culture was prepared for each test organism by inoculation of 100 ml of TSB and overnight incubation at 28°C and 60 rpm in an environmental shaker (New Brunswick Scientific, Edison, NJ). One ml of the overnight culture was transferred to a flask containing 100 ml of TSB and incubated at 28°C and 200 rpm. The OD_{600nm} was determined using a Spectronic Genesys Spectrophotometer (Milton Roy, Rochester, NY) at periodic intervals until an absorbance OD of 0.9 to 1.0 was reached. Twenty ml of culture was then harvested by centrifugation at 10,000 x g for 5 min at room temperature. The supernatant was removed and the cell pellet was washed and centrifuged two times with PB. After the final wash the supernatant was removed and the pellet was resuspended in 20 ml of PB. Cells were electronically enumerated using a Beckman Coulter Multisizer 3 with a 30 μm diameter aperture (Beckman Coulter, Miami, FL). Dilutions of cells were suspended in Isoton II (Beckman Coulter), an electrolyte solution. Each bacterial suspension was electronically enumerated 5 times. Coincidence correction was applied to values by the instrument software. Values were averaged to determine cells/ml, and each analysis was repeated for 3 replicate suspensions to determine the variability of the method.

Preparation of Universal QPCR Standards

S. oneidensis, *P. aeruginosa*, *C. fimi*, and *B. atrophaeus* were used to make universal QPCR quantitation standards. After electronic enumeration each organism was serially diluted 10-fold to concentrations ranging from 1.6×10^0 to 1.6×10^5 cells. The DNA from 500 µl aliquots of each dilution was extracted, purified and stored at - 70°C.

DNA Extraction and Purification

Five ml aliquots of environmental air samples were concentrated with a sterile 13-mm-diameter 0.65-µm-pore-size HA filter membrane (Millipore Corp., Bedford, MA), and resuspended in 500 µl of PB. Air samples, standards and cell cultures were extracted by heat and enzymatic treatment, a process previously developed by Buttner *et al.* (2001). Briefly, a 500 µl aliquot of each sample was treated with sodium dodecyl sulfate (SDS, final concentration, 0.5%, Sigma) and proteinase K (final concentration, 20 µg/ml, Sigma), heated at 50°C for 5 minutes and boiled for 15 minutes. Samples were chilled for 2 minutes followed by addition of bovine serum albumin (BSA) (final concentration, 0.05%, Sigma) and incubation in an environmental shaker for 5 minutes at 37°C and 230 rpm. The membrane was aseptically removed from environmental air samples and discarded. The extracted DNA was purified using Pellet Paint™ (Novagen, Madison, WI) according to manufacturer's instructions. After purification DNA samples were resuspended in 50 µl of Tris-EDTA buffer (TE, pH 8.0), gently mixed at room temperature for 90 minutes and stored at -70°C.

Universal Quantitative Polymerase Chain Reaction

The 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) was used for universal QPCR amplification. The target DNA codes for the 16S rRNA gene, a conserved region of bacterial DNA, producing an amplicon length of 466-bp. Universal primers and probe sequences are shown in Table 2. Universal primers were obtained from Operon Technologies (Huntsville, AL) and the probe was obtained from Applied Biosystems.

Table 2. Universal primers and probe used for universal QPCR analysis. Nadkarni *et al.* (2002).

Forward Primer	5'-TCCTACGGGAGGCAGCAGT-3'
Reverse Primer	5'-GGACTACCAGGGTATCTAATCCTGTT-3'
TaqMan Probe	6-FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA-3'

The amplification conditions for a 25 µl total reaction volume were 5 µl DNA template, 1X Universal Master Mix (Applied Biosystems), 0.1% BSA, 0.2 µM forward primer, 0.5 µM reverse primer, 0.15 µM probe, and sterile nuclease-free water (Promega, Madison, WI). Standard Mode was selected on the Applied Biosystems 7900HT PCR system. Cycling conditions were 50°C x 2 min, 95°C x 10 min, followed by 40 cycles of 95°C x 15 sec and 60°C x 1 min. The results were analyzed by the instrument software producing a standard curve of C_T versus concentration. The C_T is the QPCR cycle where fluorescence is first detected crossing the threshold and is inversely proportional to the concentration of the DNA. Concentrations for laboratory samples and environmental air samples were determined by using the equation for the composite standard curve. All primer and probe sequences and amplification and cycling conditions for universal QPCR

were previously determined (Buttner and Cruz, 2006). Negative controls were included in all universal QPCR assays and consisted of nuclease-free water (Promega). Positive controls consisted of universal QPCR standards with known concentrations as described above.

Determining Inhibition of Environmental Samples

To determine if environmental samples contained inhibitors a positive internal control (IPC) was included with all environmental air samples (IPC-VIC probe; Applied Biosystems). The IPC contains a VIC-labeled probe, DNA and primers. The universal QPCR amplifies a known concentration of IPC DNA along with the sample DNA. Inhibition is determined by noticeable changes in amplification of IPC DNA.

Statistical Analysis

Statistical analyses were conducted using SPSS v 14.0. A composite standard curve was developed by plotting the C_T value versus the log of the concentration of the universal QPCR standards. A linear regression was applied to the best fit line to develop the equation for the composite standard curve to be applied to laboratory samples and environmental air samples. A two way analysis of variance (ANOVA) was calculated to determine if there was a significant interaction between organism and analysis method. This was performed to determine if methods could be compared statistically in the absence of organism. A Tukey's post hoc test was performed to identify significant differences between methods. For laboratory experiments a paired t-test was conducted to determine significant differences between electronic enumeration and universal QPCR

concentrations. Environmental air samples were tested by a Shapiro-Wilks test to determine if data were distributed normally. The data from these samples were then analyzed statistically by a paired t-test comparing microscopy and universal QPCR.

CHAPTER 3

RESULTS

Evaluation of Universal QPCR Standards

Universal QPCR standards were developed to quantify total bacteria in environmental air samples and laboratory samples. Each of the standards, 1.6×10^5 to 1.6×10^0, was amplified by real-time QPCR using universal primers and probe (n=5). A standard curve was obtained for each organism, as well as a 95% confidence interval for estimation (Fig. 1). The standard curves of each organism were plotted and compared to each other to show the difference in amplification efficiency. Amplification of *B. atrophaeus* standards showed poor sensitivity compared to the other 3 organisms (Fig. 1). To correct for this, a composite standard curve was determined (Fig. 2) and the equation for the straight line was calculated by conducting a linear regression of the best fit line (Fig. 3). The equation for the composite standard curve was applied to environmental air samples and laboratory samples to calculate concentrations. The sensitivity was determined for each of the methods by comparing the lower detection limits of each assay (Table 3). Culture has a theoretical LDL of 1 cell/ml. However, culture can only detect viable cells and cannot determine total bacterial counts thus having poor sensitivity when compared to total count methods. The LDL for microscopy was calculated at

1.7 x 10^4 cells/ml, indicating this method has poor sensitivity. Universal QPCR was able to detect 1.6 x 10^0 cells/ml whereas the composite standard curve, developed for universal QPCR, had a sensitivity of 1.22 x 10^2 cells/ml.

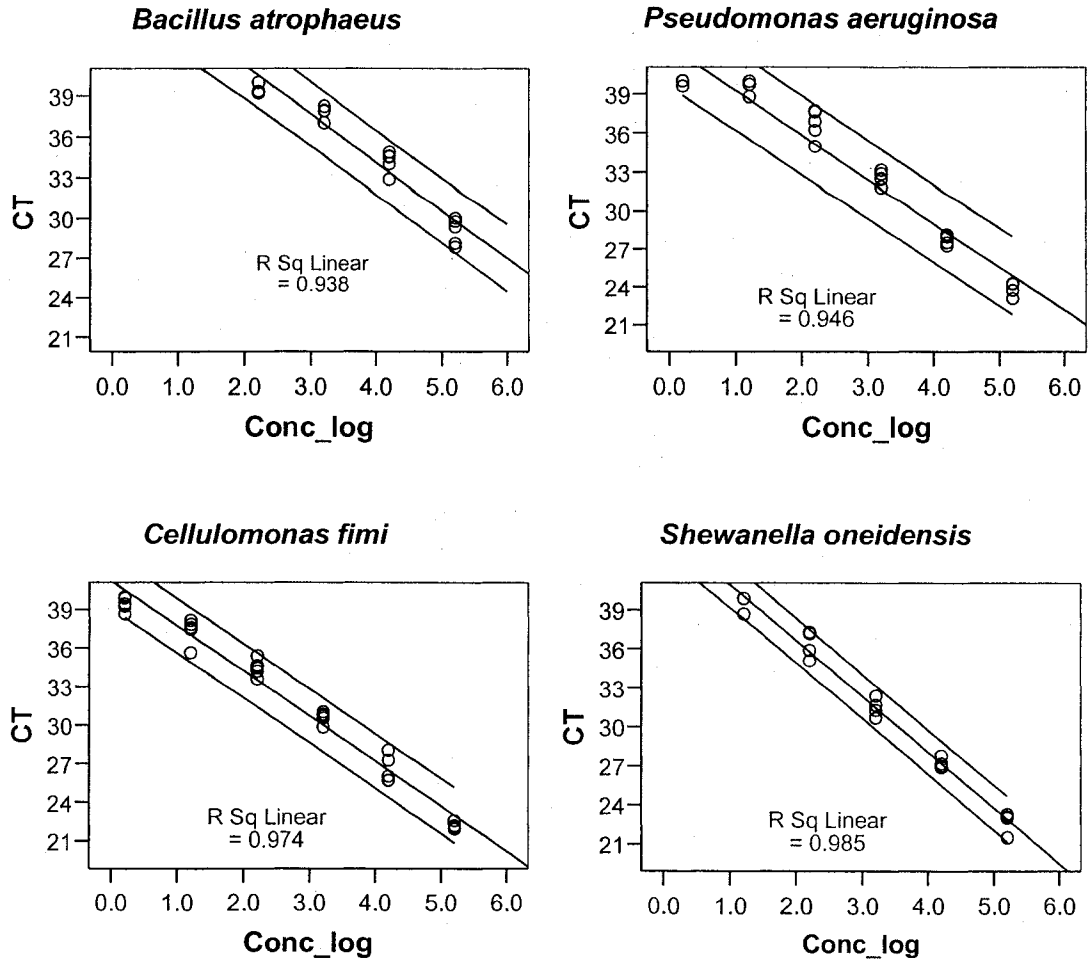

Figure 1. Universal QPCR results with C_T values of the four test organisms for each standard dilution, 1.6 x 10^0 to 1.6 x 10^5 cell equivalents/ml, plotted against the $\log_{10}$ of the concentration (n=4). The C_T is when fluorescence is first detected crossing the threshold during the universal QPCR assay. The concentration was determined by universal QPCR and the log was calculated. A best fit line with 95% confidence interval was applied to the data points for each of the test organisms.

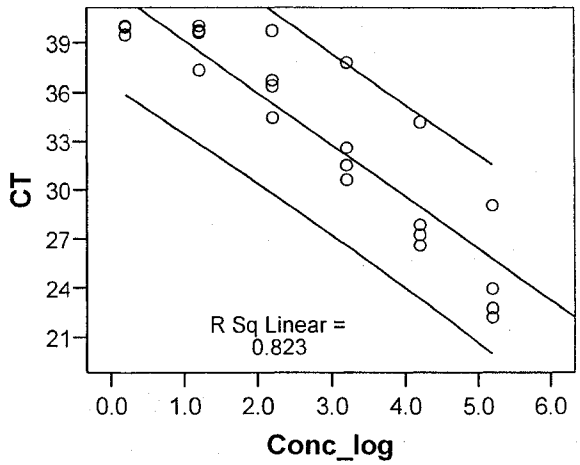

Figure 2. The composite standard curve was developed by plotting the mean C_T values of the four test organisms for each standard dilution, 1.6×10^0 to 1.6×10^5 cell equivalents/ml, against the $\log_{10}$ of the concentration (n=4) determined by universal QPCR. The C_T is when fluorescence is first detected crossing the threshold during the universal QPCR assay. A best fit line with 95% confidence interval was applied to the data points.

Formula of composite standard curve

$y = mx + b$ where, y = PCR C_T value
m = slope = -3.172
x = log of concentration
b = y-intercept = 42.318

Therefore,

$$x = \text{antilog}\ \frac{(y - 42.318)}{-3.175}$$

Figure 3. The composite standard curve equation derived from the composite of all data points from the four organisms used for universal QPCR standards.

Table 3. Comparison of lower detection limit for each method.

Method	Lower Detection Limit (cells/ml)
Culture	1
QPCR – *C. fimi*	1.6
QPCR – *S. oneidensis*	16
QPCR – *B. atrophaeus*	160
QPCR – *P. aeruginosa*	160
QPCR – composite standard curve	122
Microscopy	1.7×10^4

Methods Comparison

Pure cultures of organisms used as universal QPCR standards were used to compare three different methods: culture, microscopy, and universal QPCR to determine the variability of the methods individually and compared to each other. All methods were compared to the reference method of electronic enumeration with a Coulter Multisizer 3 (Fig. 4).

A statistically significant ($p < 0.001$) interaction was observed between the organisms and the analysis method; significance of method and organism cannot be tested independently. Therefore, an ANOVA was conducted on the three methods for each organism to determine significance of interaction. For *C. fimi* the ANOVA had a p-value of 0.006 indicating that there was a significant interaction between methods. A Tukey's post hoc test showed that there was a significant difference between microscopy and electronic enumeration ($p = 0.008$). There was no significant difference between microscopy and universal QPCR ($p = 0.014$) or between electronic counts and universal QPCR ($p = 0.839$). When the methods were compared for *S. oneidensis* by ANOVA, the

p-value was 0.085 indicating that none of the methods were significantly different. ANOVA results for *B. atrophaeus* showed significant interaction of methods ($p < 0.001$). A Tukey's post hoc test was conducted showing significant difference between electronic enumeration and universal QPCR ($p < 0.001$) and microscopy and universal QPCR ($p < 0.001$) supporting the evidence of poor amplification of *B. atrophaeus*. An ANOVA was conducted on *P. aeruginosa* which showed a significant interaction between the methods ($p = 0.009$). In addition, a Tukey's post hoc test was performed which showed electronic enumeration and universal QPCR being significantly different ($p = 0.007$). For this organism microscopy and universal QPCR ($p = 0.113$) and microscopy and electronic counts ($p = 0.115$) were not significantly different. The method of universal QPCR was shown to be a less accurate method for *B. atrophaeus* and *P. aeruginosa* based on the Tukey's post hoc results. However, for *C. fimi* and *S. oneidensis* no significant differences were seen between universal QPCR and electronic enumeration, the reference method, suggesting that it was an accurate method for determining cell concentrations.

Figure 5 shows the variability of the methods with bar heights representing the span of the 95% confidence interval. The means 95% confidence interval (C.I.) were plotted for each method for each organism (Fig. 5). When the 95% confidence intervals cover the same range in concentration it suggests that the means are not different. *C. fimi* had overlapping of the concentrations for all three methods suggesting that there was not

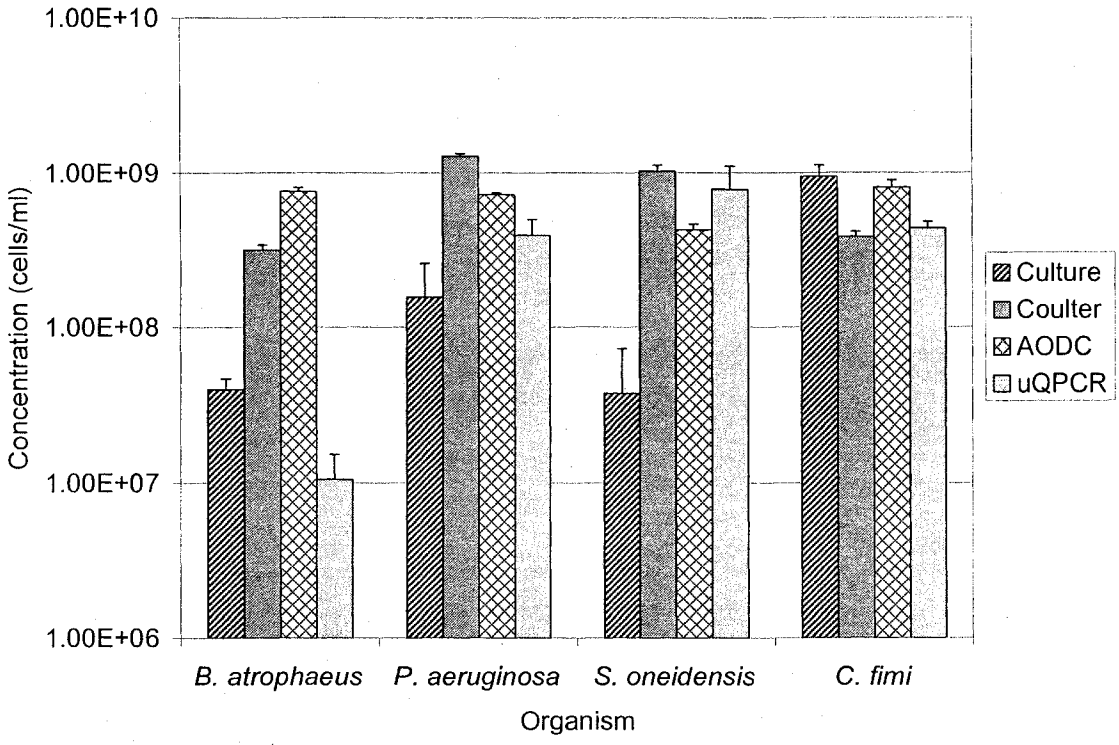

Figure 4. Results comparing analysis methods for each test organism to determine if any significant differences exist between methods. Bar heights represent the mean of three replicates (culture and AODC), mean of fifteen replicates (Coulter), and eight replicates (universal QPCR). Error bars represent the standard error of the means (uQPCR = universal QPCR, AODC = microscopy, and Coulter = electronic enumeration).

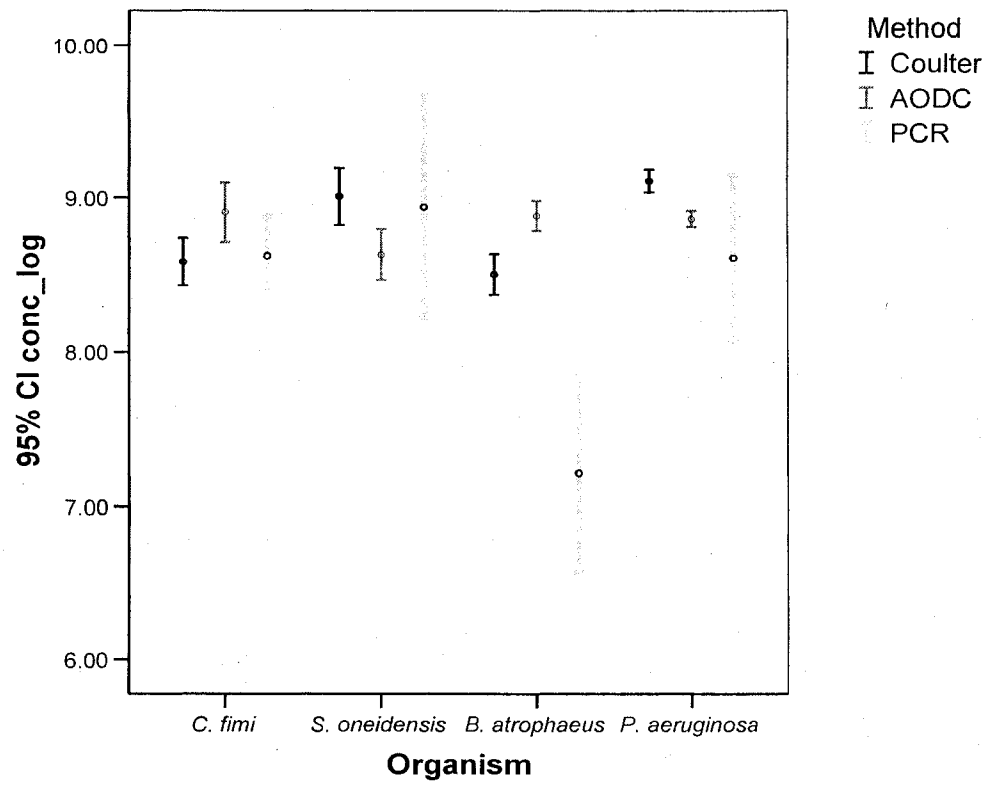

Figure 5. Comparison of the $\log_{10}$ of the means with a 95% confidence interval for electronic enumeration (Coulter), microscopy (AODC) and universal QPCR (PCR) for each test organism.

a significant difference between methods. *S. oneidensis* and *P. aeruginosa* exhibited similar results and did not show overlapping of the electronic enumeration and microscopy methods, suggesting significant differences between these methods; however, they are both overlapped by universal QPCR, indicating no difference with this method. *B. atrophaeus* does not exhibit overlapping between any methods suggesting that the means are very different for this organism. Universal QPCR has the greatest variability with all four organisms due to large error bars. Microscopy seems to have the lowest variability due to the tightness of the error bars.

Evaluation of Universal QPCR

Quantitative PCR was evaluated by comparing it to electronic enumeration by using mixed cultures of test organisms that were not used in preparing the PCR standards (Table 4). QPCR results showed that the high concentration of mixed cultures had an average concentration of $1.55 \times 10^8 \pm 3.26 \times 10^7$ templates/ml. Average concentrations of the pure cultures ranged from $2.04 \times 10^7 \pm 1.23 \times 10^7$ templates/ml to $6.90 \times 10^8 \pm 3.64 \times 10^7$ templates/ml. Mid-range mixed cultures had average concentrations of $2.39 \times 10^6 \pm 7.32 \times 10^5$ templates/ml. Average concentrations of the pure cultures ranged from $1.14 \times 10^6 \pm 2.04 \times 10^5$ templates/ml to $5.14 \times 10^6 \pm 7.87 \times 10^5$ templates/ml. Low-range mixed cultures had average concentrations of $1.68 \times 10^3 \pm 1.06 \times 10^3$ templates/ml. Average concentrations of the pure cultures ranged from $1.27 \times 10^3 \pm 6.49 \times 10^1$ templates/ml to $1.08 \times 10^4 \pm 7.36 \times 10^3$ templates/ml (Table 4).

A paired t-test was performed to determine if there was a significant difference between the electronic enumeration concentrations of the mixed and pure cultures and universal QPCR concentrations of the same cultures. Based on these data, there is evidence to suggest that the electronic enumeration and the universal QPCR concentrations were not equal ($t = -5.205$, $df = 35$, $p < 0.001$). However, they are highly correlated (correlation coefficient = 0.971), with a strong linear relationship (Fig. 6). This suggests that the QPCR values and the electronic enumeration calculations have a significant linear association even though their distributional means were not equal.

Table 4. Universal QPCR analysis of mixed culture using 3 test organisms at equal concentrations as determined by electronic particle counting (Coulter). Pure cultures of each organism used in the mixed culture were also analyzed by universal QPCR for comparison to determine the effect on the universal QPCR assay. The percent difference between Coulter and universal QPCR was calculated. (Mixed cultures (n=7) and pure cultures (n=2)).

Culture	Coulter Concentration (cells/ml)	Universal QPCR Concentration (cell equivalents/ml), ± 1 S.E.	% Difference between Coulter and QPCR
Mixed culture	3.00×10^7	$1.55 \times 10^8 \pm 3.26 \times 10^7$	81
E. coli	3.00×10^7	$8.17 \times 10^7 \pm 3.64 \times 10^7$	63
S. aureus	3.00×10^7	$6.90 \times 10^7 \pm 5.78 \times 10^8$	57
B. cereus	3.00×10^7	$2.04 \times 10^7 \pm 1.23 \times 10^7$	32
Mixed culture	3.00×10^5	$2.39 \times 10^6 \pm 7.32 \times 10^5$	87
E. coli	3.00×10^5	$1.14 \times 10^6 \pm 2.04 \times 10^5$	74
S. aureus	3.00×10^5	$1.75 \times 10^6 \pm 4.44 \times 10^5$	83
B. cereus	3.00×10^5	$5.14 \times 10^6 \pm 7.87 \times 10^5$	94
Mixed culture	3.00×10^3	$1.68 \times 10^3 \pm 1.06 \times 10^3$	44
E. coli	3.00×10^3	$1.27 \times 10^3 \pm 6.49 \times 10^1$	56
S. aureus	3.00×10^3	$2.67 \times 10^3 \pm 3.41 \times 10^2$	11
B. cereus	3.00×10^3	$1.08 \times 10^4 \pm 7.36 \times 10^3$	72

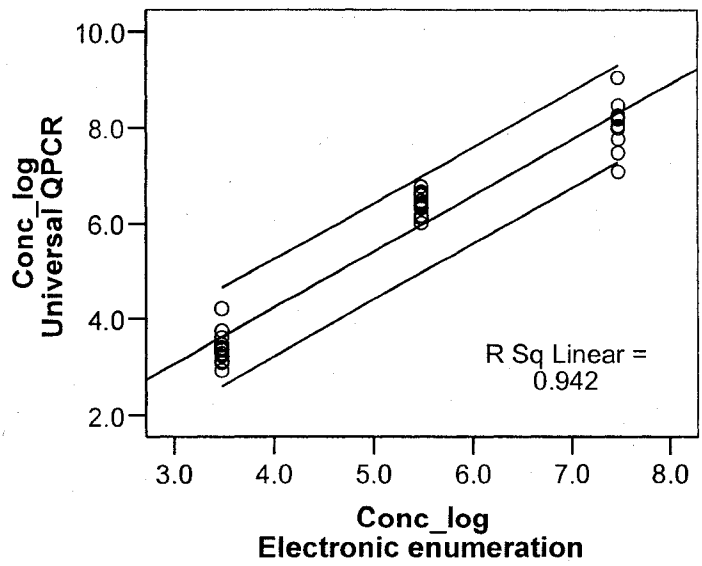

Figure 6. The $\log_{10}$ of the concentration (cell equivalents/ml) for universal QPCR was plotted against the $\log_{10}$ of the concentration (cells/ml) for electronic enumeration for mixed cultures containing *E. coli*, *B. cereus*, and *S. aureus*. A best fit line with a 95% confidence interval was applied to the data points. The methods of electronic enumeration and universal QPCR had a strong linear relationship and were highly correlated (correlation coefficient = 0.971). (Universal QPCR mixed cultures (n=7) and electronic enumeration (Coulter) (n=3)).

Analysis of Environmental Air Samples

The environmental air samples were analyzed by culture, microscopy and universal QPCR. Electronic enumeration was not used in the analysis because particles that are captured along with the bacteria cannot be differentiated from bacteria. Culture analysis was performed on environmental air samples (Table 5), but because not all bacteria are able to form colonies when cultured the results were not included in the statistical analysis.

Table 5. Culture results from environmental air samples (n=22). Lower detection limit for culture is defined as 1 CFU/ml. (CFU = colony forming units).

Sample Name	CFU/ml
113006A	< 1
113006B	3.9×10^1
120606A	1.5×10^0
120706A	3.1×10^2
120706B	7.7×10^2
121106A	1.5×10^1
121106B	3.7×10^1
030907a1	3.0×10^0
030907a2	6.0×10^0
030907b1	1.0×10^0
030907b2	3.0×10^0
030907c1	5.0×10^0
030907c2	2.0×10^0
032307a1	2.0×10^1
032307a2	3.0×10^0
032307b1	2.0×10^0
032307b2	4.0×10^0
032607a1	1.0×10^1
032607a2	2.5×10^1
032607b1	1.5×10^1
032607c1	5.5×10^1
032607c2	1.3×10^2

A Shapiro-Wilks' test was performed on the universal QPCR and microscopy results to determine if the data were normally distributed. Results of the test indicated that data for universal QPCR ($p = 0.525$) and microscopy ($p = 0.159$) were normal. To statistically test normal data, a paired t-test was performed to compare microscopy and universal QPCR. The p-value was < 0.001 concluding that the mean for microscopy was greater than the universal QPCR mean. This suggests that microscopy was able to determine higher total bacterial concentrations in air samples than universal QPCR (Fig. 7).

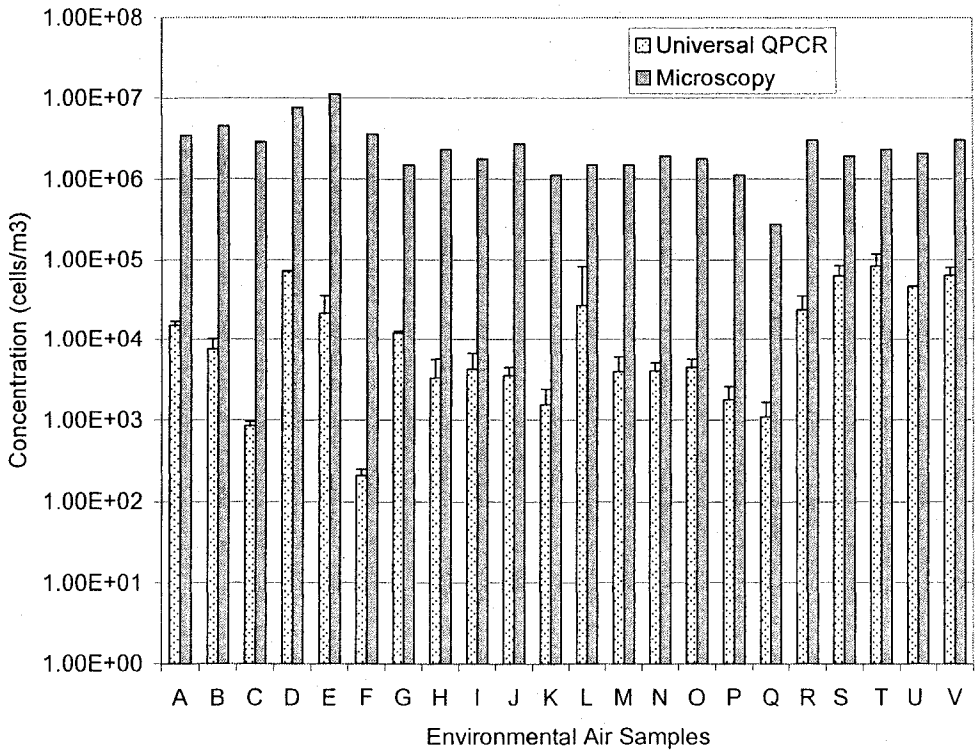

Figure 7. Comparison of universal QPCR and microscopy concentrations from environmental air samples (n=22) reported as cells/m^3. Bar heights represent the means of four replicates (universal QPCR) and only one replicate for microscopy ± 1 S.E. Due to limited sample volume duplicate microscopy analysis was not performed.

An internal positive control (IPC) PCR was conducted on all environmental air samples to determine if there were inhibitors present. Results showed that inhibitors were not present in environmental air samples (data not shown).

CHAPTER 4

DISCUSSION

Evaluation of Universal QPCR Standards

Quantitation standards are needed to adequately measure total airborne bacterial concentrations with universal QPCR. Due to the varied amplification of bacteria, noted during previous studies (Buttner and Cruz, 2006; Nadkarni *et al.*, 2002), a composite standard curve of four test microorganisms was developed in this study. When the standard curves were developed for each test organism independently, variation of amplification was noted (Fig. 2). This exhibits the same finding that was seen during the specificity testing where it was noted that organisms had different amplification efficiencies with the universal primers and probe (Buttner and Cruz, 2006). This could be due to the number of target sequences (rDNAs) varying among bacterial species in the number of copies per genome as well as with the growth phase of the harvested cell (Lyons *et al.*, 2000; Klappenbach, 2001). Multiple copy numbers seen during rapid growth are due to increased cellular components produced during cell replication.

Lyons *et al.* (2000) successfully used a composite standard curve of four organisms to quantify mixed samples when testing total bacteria in dental plaque. Therefore, this method was adopted with modifications in this study to correct for variation in amplification efficiencies. Cultures were harvested at approximately the same growth stage to maintain consistency. Any variations in copy number should have been

normalized during the electronic enumeration and DNA extraction. By using electronic enumeration for standards and extracting standards and samples in the same manner, both multicopy DNA and DNA losses during extraction were resolved, respectively. This would eliminate any concern due to differences in growth phase and copy number. Other studies have generated standard curves using CFU, 16S rDNA copy number or prepared from cells gathered the same way as samples being tested (Lyons *et al.*, 2000; Nadkarni *et al.*, 2002; An *et al.*, 2006). The advantage of using electronic enumeration is that it applies a total count method and does not rely on culture or rDNA copy number. A cell is counted as one cell regardless of the number of copies of the same gene. Extracting standards in the same manner as the samples compensates for losses that may occur. Therefore, when utilizing standards to enumerate environmental samples a more accurate method of quantitation results regardless of cell viability or copy number.

The hypotheses proposed in this study were that universal QPCR could provide a more accurate and sensitive method for measuring airborne bacteria than the traditional methods of culture analysis and microscopy. These hypotheses were tested with the standards developed to quantitate environmental air samples. The first hypothesis, providing a more sensitive method, was determined by calculating and comparing the lower detection limits of universal QPCR, microscopy and culture analysis. Table 3 showed the values for each method and the conclusion was that universal QPCR is a more sensitive detection method for measuring total bacterial concentrations in air samples.

The second hypothesis, providing a more accurate method, was tested by comparing universal QPCR to microscopy and culture analysis. A statistical analysis showed that

B. atrophaeus and *P. aeruginosa* had significant differences between the reference method, electronic enumeration, and universal QPCR. *B. atrophaeus* showed poor amplification for reasons unknown at this time. This lowered the accuracy of universal QPCR. However, *C. fimi* and *S. oneidensis* showed no differences between universal QPCR and electronic enumeration concluding universal QPCR to be a fairly accurate method for measuring airborne bacteria.

Methods Comparison

Four methods were tested in this study; culture, microscopy, electronic enumeration and universal QPCR. Although, electronic enumeration, used as a reference method, is very accurate it cannot differentiate between bacterial microorganisms and other particulate; therefore, it cannot be used for environmental sample analysis. However, it was used to determine bacterial cell suspension concentrations and as a reference method for comparison in laboratory experiments.

Culture was not directly comparable to microscopy and universal QPCR even with pure cultures. Some cells may be viable but not culturable (VBNC) due to stressful conditions such as aerosolization, sampling stresses, nutrient deficiencies or competition on the culture media and not detected. Microscopy and universal QPCR are other methods that are available to quantitate total bacteria in samples. It was observed (Fig. 5) that microscopy was better at quantitating test organisms than universal QPCR. Due to a significant interaction ($p<0.001$) between organism and methods a statistical analysis could not be performed independently on laboratory methods.

According to the *E. coli* variability map of the 16S rRNA gene provided by the European Ribosomal RNA database over 10% of the nucleotides in the 16S rRNA gene are totally conserved but none of them form a continuous conserved region for universal priming (Baker *et al.*, 2003). This makes designing a primer difficult, and the likelihood of unamplified products increases due to probable mismatches.

Microscopic analysis using the direct count method with the Acridine Orange stain was shown to be slightly more effective in determining the concentration of test organisms (Fig. 4). However, microscopy can be overestimated due to auto fluorescence or nonspecific staining of particulate (Kepner and Pratt, 1994; Terzieva *et al.,* 1996). In addition, bacterial cells can be "rafted" or attached to debris (Maron *et al.*, 2005) making the bacterial cell harder to see, leading to inaccurate total counts of environmental samples.

Evaluation of Universal QPCR

To determine if there was an interaction of mixed cultures with the universal QPCR assay, three test organisms that were not used as universal QPCR standards were equally mixed at three concentrations and analyzed by universal QPCR. The results indicated that although the electronic enumeration concentrations and the universal QPCR concentrations were not equal they had a significant linear association. Although the universal QPCR concentrations were higher than the electronic enumeration concentrations they were not extremely different. Farrelly *et al.* (1995) noticed in their PCR amplifications that the pairing of *Bacillus subtilis* and *Thermus thermophilus* produced unexplained high deviations from the predicted values and that there is an

intrinsic but unknown feature that is causing this result. The Farrelly *et al.* (1995) study suggested possible factors resulting in these deviations, including 16S rDNA proportions, G+C content, and location of *rrn* operons on the genome. However, knowing this still does not provide exact predictions. It has been observed that organisms with a high G+C content such as Actinobacteria are underrepresented or even absent in 16S rRNA PCR based studies (Hill *et al.*, 2006). *C. fimi*, which was used as a universal QPCR standard in this study, belongs to the Phylum Actinobacteria and amplified well in universal QPCR testing. In a previous study conducted by Buttner and Cruz (2006), 39 microorganisms were tested with universal primers, 6 of which were Actinobacteria. Four of the 6 Actinobacteria showed strong amplification. However, these data were derived from pure culture and may be different from those obtained from air samples. Discovery of new taxa with 16S rDNA sequences not complementary to standard universal primers suggests that current 16S rDNA libraries are not representative of true prokaryotic biodiversity (Baker *et al.*, 2003). Therefore, the variability of universal primers raises the question as to the accuracy of universal QPCR.

Analysis of Environmental Air Samples

There are various methods for collecting air samples that incorporate a culture based approach including liquid impingement (Grinspun *et al.*, 2007). Microbial stress during aerosolization and sampling of airborne microorganisms and the violent motion in the liquid during collection may affect the viable count in an impinger sample in a time-dependent manner but most damaged cells could recover (Terzieva *et al.*, 1996). However, total count analysis methods are still necessary.

In this study microscopy showed higher concentrations than universal QPCR when analyzing environmental samples (Fig. 7). However, microscopy may over-estimate total bacterial counts. Microscopy is labor intensive and requires an experienced observer to differentiate between particulate and microorganisms. Autofluorescence, nonspecific staining of cellular components, and dark or shadowed cells make it difficult to correctly identify bacterial organisms (Terzieva *et al.*, 1996). This may be a reason for the higher concentrations obtained by microscopy compared to universal QPCR.

Hill *et al.* (2006) suggested that organisms such as Actinobacteria are underrepresented or nondetectable in environmental samples. *C. fimi,* a member of the Phylum Actinobacteria, was one of the four microorganisms used to make a composite standard curve in this study. DNA extracted from a pure culture amplified very well in universal QPCR assays. A previous study conducted using the universal primers and probe amplified 6 representative bacteria from the phylum Actinobacteria with C_T values ranging from 20 – 40 (Buttner and Cruz, 2006). Two of the 6 Actinobacteria had poor amplification. In addition, universal QPCR amplification of organisms retrieved by sampling may produce decreased amplification efficiencies of those organisms. This may be caused by the cell membrane becoming disrupted and internal components being destroyed (e.g., shearing of the DNA). Samples in this study were exposed to several cycles of freeze-thaw during storage and quantification by universal QPCR analysis. This may have also had an effect on bacterial DNA detection. Another source for concern is DNA degradation over time which has been reported (Josephson *et al.*, 1993).

Another factor that could pose a problem for universal QPCR is the presence of inhibitors. Inhibitors can interfere with QPCR preventing target DNA from amplifying and producing false negative results (Stetzenbach *et al.*, 2004). Although microscopy was slightly better than universal QPCR in determining sample concentrations it is labor intensive, time consuming, and expertise is needed. The advantages of universal QPCR are that it is rapid, sensitive, has a high sample throughput, and low numbers of targeted microorganisms are needed for detection (Alvarez *et al.*, 1995). The advantages of universal QPCR make it a suitable candidate for detecting bacterial concentrations in environmental air samples.

CHAPTER 5

CONCLUSION

This study focused on using universal QPCR primers and probe to quantify total bacterial concentrations in environmental air samples. While not perfect, this method was able to effectively amplify bacterial DNA from environmental air samples. Universal QPCR can be a quick and efficient method to determine if an air quality complaint or event is bacterial in nature allowing for immediate action. Additional research is needed to address the amplification efficiencies of organisms present in environmental samples.

Universal QPCR can be useful in indoor air quality surveillance. Bioterrorism and biowarfare have constantly been an issue since the attacks on the World Trade Center in New York City in 2001. Currently there are multiple sampling units placed in cities around the United States which monitor the air for certain biowarfare agents. The problem lies in the ability of these units detecting anything other than the specific agents of interest. If an unknown non-target, but pathogenic bacterial agent were to be collected by one of these units it would not be detected due to the specific detection parameters that the unit was designed to test for. Therefore, universal QPCR can be used to determine if there is a large increase in a sample, other than normal seasonal fluxes, of a bacterial nature, allowing the correct evacuation or decontamination procedures to take place.

Another use for universal QPCR is for monitoring of biocontamination. This would aid in indoor air quality investigations to determine if there is a high indoor bacterial count. Water damaged environments and/or work environments such as hospitals, dentist offices and medical clinics could benefit from this method. Industrial applications of universal QPCR include waste water treatment plants, agricultural settings, food industries, and landfills. High (undetected) concentrations of bacteria can be problematic for those that work in these environments leading to adverse health effects. Other areas of concern are enclosed spaces such as International Space Station, Shuttle, and submarines. Monitoring these spaces with a method such as universal QPCR could help identify bacterial problems that may be hazardous to the occupants.

Universal QPCR assays for detection of airborne bacteria can aid in determining the nature of an outbreak with health related illnesses. Knowing if an outbreak is caused by airborne bacteria a course of treatment can be applied sooner than waiting for culture analysis results that could take weeks if the unknown agent is able to grow when cultured.

An example would be the Legionnaire's convention of 1976. Of the 182 members of the Pennsylvania American Legion that became sick, 29 individuals died (Winn, 1988). It was not known if the mysterious affliction was chemical, bacterial or something else. If this type of event were to occur today universal QPCR could be used as a screening tool to determine if the agent was bacterial.

Although, universal QPCR, may still need improvement it is more rapid and less time involved than microscopy. There are many possible applications to using universal QPCR but further testing needs to be conducted to improve on the accuracy and sensitivity of the method. Future studies should include the development of composite standard curves with additional organisms.

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