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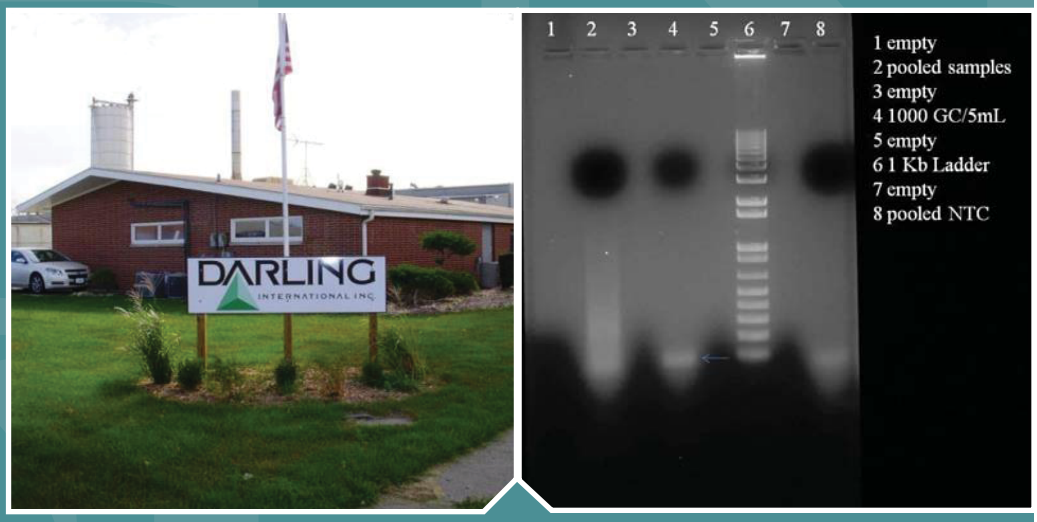
United States
Environmental Protection
Agency

Technical Report

Field Study on Cleaning a Rendering Plant Following a Foreign Animal Disease (FAD) Outbreak



Office of Research and Development
National Homeland Security Research Center

Technical Report

U.S. Environmental Protection Agency

Field Study on Cleaning a Rendering Plant Following a Foreign Animal Disease (FAD) Outbreak

U.S. Environmental Protection Agency

Office of Emergency Management
CBRN Consequence Management Advisory Team
Research Triangle Park, NC

National Homeland Security Research Center
Decontamination and Consequence Management Division
Research Triangle Park, NC

NOTICE

The U.S. Environmental Protection Agency (EPA), through its Office of Emergency Management's (OEM's) Consequence Management Advisory Team (CMAT) and Office of Research and Development's (ORD's) National Homeland Security Research Center (NHSRC), directed and managed this work through Contract Number EP-W-12-026, Task Order TO-02-011 with Dynamac Corporation. Funding for this work came through Interagency Agreement RW-12-92306101 with the U.S. Department of Agriculture. This report has been subjected to the Agency's administrative review and approved for publication. The views expressed in this report are those of the authors and do not necessarily reflect the views or policies of the Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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EXECUTIVE SUMMARY

Test Objectives

A study was conducted to evaluate cleanup of a rendering plant after its use for disposal in response to a Foreign Animal Disease (FAD) outbreak. The intent of this study was to develop recommended procedures that could be used to aid in returning a rendering plant to normal operation after use in support of an actual FAD incident.

This effort attempted to achieve three objectives:

- To generate data on fugitive emissions of a biological surrogate during the rendering process;
- To determine the effectiveness of existing plant cleaning procedures for reducing the levels of surrogate on the inside surfaces of the rendering facility; and
- To provide information that could be used to develop standard procedures for appropriately clearing a rendering facility that had been used for "disposal rendering" after an FAD outbreak, as part of returning the rendering facility back to its normal production use.

The Test Team conducted several sampling events at the Darling International (Darling) Rendering Plant located in Des Moines, Iowa, which included:

- Acquiring a series of opportunistic swab samples at the first plant visit to gain an initial insight into the culturable bacterial flora present in the plant;
- Acquiring a series of wipe samples at various locations in the plant to get a more detailed evaluation of background culturable bacterial flora present in the plant;
- Performing an initial sampling effort to focus on potential biological surrogates to use for the Cleaning/Inoculation study;
- Performing a series of laboratory spike tests involving potential biological surrogates in idealized rendering plant sampling matrices and sampling media for air and wipe samples. Based on the results of this and all previous testing, biological and nonbiological surrogates were selected for the Cleaning/Inoculation study; and
- Performing a Cleaning/Inoculation study at the rendering plant to evaluate the movement of the surrogates within the rendering process and subsequent plant cleaning procedures.

Although use of a thermophilic bacterium such as *Geobacillus stearothermophilus* as a surrogate was originally desired, a lack of ability to positively identify *G. stearothermophilus* using molecular microbiological techniques led the Test Team to select a nonthermophilic organism. Based on the initial tests, an inoculum was selected for the Cleaning/Inoculation study that was a mixture of 1E9 colony forming units (CFU) of *Bacillus atrophaeus* (aka *Bacillus globigii* or *Bg*) spores and 1.47E9 beads of Polylactic-Co-Glycolic Acid (PLGA) fluorescent microspheres, with an additional surfactant "Fluid D" per gallon of inoculum.

Over a series of weekends, the rendering plant was cleaned using cleaning procedures normally utilized by the plant. Following the plant pre-cleaning, the Cleaning/Inoculation study was then conducted in October of 2011.

Test Conclusions

The following conclusions were drawn from the Cleaning/Inoculation study:

- The results of the Cleaning/Inoculation study indicated that no *Bg* deoxyribonucleic acid (DNA) was detected in any of the sample extracts from the post-inoculation or post-cleaning surface wipes or from the air samples using various polymerase chain reaction (PCR)

techniques. A significant amount of additional effort was devoted to extracting *Bg* DNA from the samples, including the use of alternate means to amplify the *Bg* DNA and achieve detection. This additional effort was unsuccessful. Although *Bg* was possibly present in low concentrations and below the limit of detection by quantitative PCR (qPCR), nondetection by qPCR was very possibly due to inhibitors present in the sample matrices that carried over during the extraction process. This hypothesis was formulated because putative *Bg* was recoverable on brain heart infusion agar (BHIA) using nonmolecular microbiological techniques and because *Bg* DNA could be extracted from, and detected in, spiked positive controls of pristine gauze and filter matrices, as well as idealized materials similar to rendering plant sample matrices (i.e., suet, grease, and deionized [DI] water).

- Due to problems with extracting the PLGA microspheres from the sample matrix (both gauze wipes and air filters), PLGA might not be a suitable synthetic surrogate, as the microspheres become permanently immobilized in these sampling matrices. Extraction processes were ineffective at removing PLGA microspheres for quantitation by fluorometer. In addition, autofluorescence from the rendering plant sample matrices (e.g., grease, flesh, bone materials) complicated detection of PLGA microspheres via direct microscopic observation. Other PLGA microspheres with different colors that may not autofluoresce at the same wavelength as the rendering sample matrices may be available. There were two issues with the PLGA microspheres: immobilization on sampling materials and detection interference caused by rendering materials. Other sampling matrices may possibly yield better results with PLGA microspheres.
- Both PLGA and PCR analysis of rendering matrices proved to be difficult. Strides were certainly made to help identify which analysis methods might work better to overcome interferences such as hair, grease, and bone fragments. However, questions linger about qualitative and quantitative analysis of rendering plant samples in the future. In addition, this study raised questions concerning identification and use of a suitable surrogate and the materials that would be necessary to acquire and analyze samples from an environment containing considerable background biological microbes.
- Using nonmolecular microbiological culture techniques, viable bacteria very similar to the *Bg* positive control colony morphology were recovered from eleven of the test sample extracts (five contained putative *Bg* in quantities greater than the limit of quantitation [LOQ]).
- Based on results obtained from nonmolecular biological culture techniques, routine plant cleaning procedures may potentially result in an approximately 1-log reduction in pathogen loading within the potentially contaminated areas of the plant. This result is consistent with results from previous systematic studies examining the effectiveness of different steps of a multi-step cleaning/disinfection process that showed a 1-4 log reduction from individual cleaning/disinfection steps. The plant cleaning procedures used in this study utilized hot water and steam, a combination that would have been expected to remove contamination from surfaces and transfer any removed contamination into the rinse water going into the drains, as opposed to actually killing any surrogate organisms that would have existed in the rinsate. Hot water would not have killed the surrogate spores used in these tests, but may possibly kill some FAD viral agents.
- The cleaning process using the steam and hot water has the potential to spread the contaminant throughout the plant, even if the cleaning process results in a reduction in the overall levels of contamination. It is not entirely clear as to whether this dispersion of contaminant is the result of plant personnel tracking the surrogate to various locations within the plant or due to aerosol transport. High pressure spraying operations have been shown to result in aerosol transport of spores. However, no air samples exhibited any *Bg,* either through PCR analysis or by examination of colony morphology.

This study highlights the need for analytical methods that are compatible with the matrices found in rendering facilities.

Recommendations for Future Rendering Plant Sampling/Analytical Efforts

The information that was obtained from this study leads to many questions about the sampling and analysis of the rendering plant matrices and air samples. The study revealed that more work should be done to determine how to sample in a rendering facility environment and to analyze the resulting extracts.

- Both wipe samples and swabs were used for sampling in this study because of the harsh environment (i.e., rough, grimy surfaces) of a rendering plant. Swab samples were negatively impacted by the rough surfaces in a rendering environment, and a single large particle could potentially bias a swab sample. While wipe samples certainly could collect more material, the amount of material collected by a wipe could require multiple dilutions during the biological analysis portion of the study. Also, the materials used in wipe samples interfered with the identification of the PLGA microspheres; i.e., PLGA microspheres became permanently immobilized in the sampling matrices.
- Sample dilution might be a better alternative for these sample matrices, or a more desirable solution for the end users, but testing would be necessary to determine the optimal dilution to overcome PCR inhibition without significant loss of target DNA. However, dilution comes at the expense of sensitivity; it is not clear whether a different/additional purification step would be more advantageous than dilution of the inhibitor.
- Newer DNA extraction methods that have recently been developed have shown promise in the ability to extract DNA from complex matrices and may be useful to test on rendering samples.
- Due to the difficulty of extracting *Bg* DNA from the sample matrices coupled with the success of using nonmolecular microbiological techniques to identify putative *Bg* colonies on heat-shocked samples, the initial desire for a thermophilic bacterium (e.g., *G. stearothermophilus*) to use as a potential biological surrogate for rendering plant studies should be revisited. Results of this study as well as a subsequent literature review indicated that further work on *G. stearothermophilus* may require construction of *Geobacillus* genus-specific (GEOBAC) primers specific to the *Geobacillus* genus based on internal transcribed spacer (ITS) sequences.
- Given that many FADs of interest are viral in nature, development of methods to extract viral DNA from rendering plant matrices may be necessary to show that there is no residual viral loading in the plant following cleaning procedures or at least that viral loading is below levels pre-determined by the Incident Commander.
- The results of the analyses indicated that PLGA microspheres may not be a suitable synthetic surrogate. The microspheres appear to become immobilized in the sampling matrices, and the particles autofluoresce at a wavelength similar to hair and bone fragments. This behavior makes it difficult to distinguish the PLGA spheres from background. Also, the extraction processes were ineffective at removing PLGA microspheres for quantitation by fluorometer, and autofluorescence from the sample matrices complicated detection of PLGA microspheres via direct microscopic observation. Other variants of the PLGA microspheres may exist that neither autofluoresce at the same wavelengths as the sampling matrices nor become immobilized in the wipe gauze or air filter materials.

Based on the results of the sampling and methods development work that has been done, an

ideal surrogate for use in the field test does not appear to exist. Tradeoffs must be taken into account and a balance struck to pick the best available surrogate given the amount of information that is currently available.

Recommendations for Developing Plant Cleaning Procedures Following Use for Disposal Rendering as Part of an FAD Response

The results from this study suggest that the development of standard operating guidelines to address the cleaning of a rendering plant following its use for disposal of animal carcasses as part of an FAD response would include several distinct steps, with precautions being taken to minimize movement of contamination. Due to the size of a typical rendering plant, the diversity of process equipment in the plant, and the level of dirt and grime on many plant surfaces, it is unlikely that fumigation would be recommended for the plant decontamination without data first becoming available to assess decontamination efficacy and potential equipment damage in a rendering plant environment. Until data on fumigation of a rendering plant scenario become available, procedures using surface cleaning and subsequent disinfection may, therefore, be the most appropriate means to restore a rendering plant to normal operation following its use in an FAD response.

The purpose of this study was not specifically to develop the cleaning guidelines, but to develop information that could be used by the rendering industry and agricultural emergency response authorities to develop guidelines that could be used to restore a rendering plant to normal operation following its use in an FAD response.

The following suggestions are offered for inclusion in plant cleaning guidelines:

- Due to the size and diversity of materials of construction in and around the rendering plant and its various process units, as well as the nature of plant operations, there are abundant opportunities to result in the buildup of a potentially significant quantity of dirt, grime, grease, and organic matter on many plant surfaces. This buildup is likely to occur over a period of time significantly longer than the time that the plant would likely be used for disposal rendering. Subsequent cleaning operations following the use of the plant for disposal rendering would be greatly facilitated if the plant were to be cleaned prior to being used for disposal rendering. This prior cleaning may present a logistical challenge due to the lead time associated with bringing in a commercial cleaning operation. However, removal of accumulated grime, dirt, and organic matter prior to potentially contaminating the plant with an FAD pathogen may greatly simplify later cleaning and decontamination operations.
- Due to the potential for transport of contamination throughout the plant due to the activity of the plant personnel, establishing contaminant control procedures for plant workers prior to delivery of any contaminated materials to the plant may be very important. Contaminant control procedures may include such considerations as:
    - Establishing egress pathways for workers to pass from areas of lower likelihood of contamination to areas of higher likelihood of contamination;
    - Dividing work duties and shift schedules so that workers performing activities in areas of lower likelihood of contamination do not enter areas of higher likelihood of contamination;
    - Establishing procedures for donning and doffing clothing and personal protective equipment (PPE) to minimize contaminant spread; and
    - Using aerosol containment equipment (e.g., tent) at the grinding operation where the most post-inoculation putative positive surrogate samples were observed.

- Due to the potential for cleaning operations to spread contamination around the plant to areas that may previously not have been contaminated, a multi-step (possibly three distinct steps) cleaning/decontamination process, done in a systematic approach with runoff control, appears to be the most effective way to clean the plant for restoration to normal operation. Initial cleaning steps may include such activities as low pressure washing, steam cleaning, and brushing. Minimization of the use of high pressure washing may minimize aerosol transport of potential contaminants.
- The multi-step cleaning/decontamination process might be a three-step process that starts with cleaning only the potentially most heavily contaminated portions of the plant, rather than the entire plant. This initial cleaning might focus on removal of organic matter, particularly on the tipping floor, in the feed hopper, the grinder, and on the auger ramps that lead into the cooker, along with the walls and floors in those areas of the plant. This initial cleaning should be staged to move the potentially contaminated materials eventually into the cooker or the drains, such as by cleaning in the following sequence:
    - Tipping floor area walls;
    - Tipping floor;
    - Feed hopper;
    - Grinder; and
    - Augers and ramps.
- During this initial cleaning operation, plant personnel movement from the areas being cleaned to other plant areas that may not be as contaminated should be minimized.
- Utilizing the cooker where possible to process potentially contaminated materials may minimize further contamination of the areas outside the plant.
- Where the cooker cannot be used to process potentially contaminated materials, the remainder may be diverted into the drains, so that runoff can be collected and treated separately.
- Once the heaviest loading of organic matter has been removed from the surfaces in the areas of the plant that have the highest likelihood of contamination (i.e., tipping floor, grinder, feed augers), subsequent cleaning operations should be initiated. These subsequent cleaning steps may include a second pass through the entire plant using steam, detergents, and low pressure spraying of water, with special attention being given to the drain areas, where rendering material may accumulate. A final cleaning step that involves the use of disinfectants that have been registered for use with the FAD organism of interest would then be performed.
- Water and other runoff that is collected in the drains should be treated to kill the FAD pathogen prior to discharge. This step is likely to vary significantly from rendering plant to rendering plant and may require concurrence by permitting authorities who regulate water discharges from the plant.

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ACRONYMS AND ABBREVIATIONS

°C	Degree(s) Celsius
°F	Degree(s) Fahrenheit
ABI	Applied Biosystems, Inc.
APHIS	(USDA) Animal and Plant Health Inspection Service
ATCC	American Type Culture Collection
Bg	*Bacillus atrophaeus* aka *Bacillus globigii*
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion Agar
BLASTn	Nucleotide Basic Local Alignment Search Tool
bp	Base Pair(s)
CBRN	Chemical, Biological, Radiological, and Nuclear
CFU	Colony Forming Unit(s)
cm	Centimeter(s)
CMAT	(EPA) Consequence Management Advisory Team
Ct	Cycle Threshold
DATS	(EPA) Decontamination Analytical and Technical Services contract
DI	Deionized
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
EDTA	Ethylenediaminetetraacetic acid
EPA	U.S. Environmental Protection Agency
FAD	Foreign Animal Disease
ft	Foot/Feet
g	Gram(s)
gal	Gallon(s)
GC	Gene Copies
GEOBAC	*Geobacillus* genus-specific primers
HF	High Fidelity
hr	Hour(s)
hsDNA	Herring Sperm Carrier DNA
ID	Identification(s)
in	Inch(es)
IPC	Internal Positive Control
ISPs	Ion Sphere Particles

ITS	Internal Transcribed Spacer
LOD	Limit of Detection
LOQ	Limit of Quantitation
Lpm	Liter(s) per minute
MC	Multicomponent
MCE	Mixed Cellulose Ester
mg	Milligram(s)
min	Minute(s)
mL	Milliliter(s)
mm	Millimeter(s)
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer(s)
NCBI	National Institute of Health's National Center for Bioinformatics
ND	No Data
ng	Nanogram(s)
nk	Number of voltage ramp steps to reach Run Voltage
nm	Nanometer(s)
NTC	No Template Control
NHSRC	(EPA) National Homeland Security Research Center
NRF	National Response Framework
OEM	(EPA) Office of Emergency Management
ORD	(EPA) Office of Research and Development
PBS	Phosphate Buffered Saline
PC	Positive Control(s)
PCR	Polymerase Chain Reaction
PLGA	Polylactic-Co-Glycolic Acid
PPE	Personal Protective Equipment
PVA	Polyvinyl Alcohol
QAPP	Quality Assurance Project Plan
QA/QC	Quality Assurance/Quality Control
QN	Qiagen Neat
qPCR	Quantitative PCR
QV	Quality Value
R&D	Research and Development

R^2	Statistical correlation coefficient
rRNA	Ribosomal Ribonucleic Acid
rtp	Replication Termination Protein
RTP	Research Triangle Park
RT-PCR	Real-time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
TBD	To Be Determined
TE	Tris ethylenediaminetetraacetic acid
TSA	Tryptic Soy Agar
USDA	U.S. Department of Agriculture

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1. INTRODUCTION

1.1 Introduction

Rendering is one of the technologies that could potentially be used to dispose of large numbers of animal carcasses generated during a response to a foreign animal disease (FAD) outbreak [1]. There are currently approximately 300 rendering facilities in North America [2]. However, guidance is not available on restoring a rendering plant to normal operation following its use for disposal as part of an FAD incident response. Therefore, in collaboration with the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (USDA/APHIS), Darling International, Inc., and the National Renderer's Association, the U.S. Environmental Protection Agency (EPA) conducted a study to evaluate fugitive emissions of a biological agent surrogate released from a rendering process and subsequent cleanup procedures. For this project, the Test Team (composed of personnel from EPA's National Homeland Security Research Center (NHSRC), EPA's Office of Emergency Management's (OEM's) Chemical, Biological, Radiological, and Nuclear (CBRN) Consequence Management Advisory Team (CMAT) agreed upon several objectives:

- To generate data on fugitive emissions of a biological surrogate from the rendering process;
- To determine the effectiveness of plant cleaning procedures for reducing the surrogate levels on the inside surfaces of the rendering facility; and
- To provide information that could be used to develop standard procedures for appropriately cleaning of a rendering facility that has been used for "disposal rendering" after an FAD outbreak so that the rendering facility can be returned to normal production.

Note that at this point, cleanup goals were not identified; this initial effort was intended to identify potential cleanup approaches and sampling strategies to use.

Environmental characterization, decontamination, and clearance are critical components of a comprehensive public health recovery strategy in the aftermath of an FAD incident or intentional release of a biological agent. Rendering plants could play a critical role in the nation's response to an FAD event by assisting in the control of diseases and providing a mechanism to recycle usable animal carcasses to safe and usable products. The National Response Framework (NRF) [3] and the Food Safety Modernization Act [4] require multiagency participation and identify USDA as the lead agency for carcass disposal with the EPA as a support agency.

As one step towards addressing the process for returning a rendering plant to normal operation, the EPA, USDA/APHIS, and the rendering industry are working together to evaluate potential cleanup approaches. The evaluation process includes characterizing the baseline biological footprint of a rendering plant, determining a biological surrogate, performing pre-release and post-release sampling, cleaning/decontaminating the rendering facility, and performing post-decontamination sampling. The EPA's CMAT and NHSRC conducted a study to evaluate the potential for cleaning a rendering plant following its potential use for disposal of contaminated animal carcasses in response to an FAD outbreak. This study consisted of several distinct components spread out over 2010 and 2011. To conduct this study, several test events occurred at the Darling International (Darling) Rendering Plant located in Des Moines, Iowa.

Table 1 lists the various study-related events, dates on which they occurred, and the purpose of that particular component of the study. Appendix C contains a photographic log of the activities for these events.

Table 1. Summary Table of Testing

Study Event	Date(s)	Purpose
Selection of Surrogate for Field Tests		
Initial site visit and preliminary scoping samples	January 6, 2010	To view the test site and to collect a limited number of opportunistic surface swab samples for the purpose of planning the tests
Initial Plant Sampling	June 15, 2010	To collect background surface samples for the purposes of identification of background flora and initial surrogate candidates
Initial Surrogate Evaluation	October 18-20, 2010	To perform a systematic sampling effort to identify appropriate surrogate(s) for field tests
Preliminary Selection of Surrogates	December 2010	Based on initial sampling, identify likely surrogate(s) to use for later field tests
Rendering Matrix Challenge Testing	August 2011	Assess recovery of proposed surrogates *Bacillus atrophaeus* (*Bg*) and polylactic-co-glycolic acid (PLGA) from model rendering plant matrices
Final Selection of Surrogates	August 2011	Make final decision on surrogates to use for Cleaning/Inoculation Study
Cleaning/Inoculation Study		
Plant pre-cleaning	September – October 2011	Remove bulk loading of organic material from plant surfaces
Background Sampling	October 2011	Sample specific locations (surfaces and air) in the rendering plant for initial concentrations of the surrogates
Inoculation Phase Sampling	October 2011	Inoculate incoming trucks loaded with animal carcasses with *Bg* spores and PLGA microspheres. Air sampling occurred during this stage of the test.
Post-Inoculation Phase Sampling	October 2011	Sample specific locations (surfaces and air) in the rendering plant for the surrogates
Plant Cleaning	October 2011	Clean rendering plant using existing plant cleaning procedures
Post-Cleaning Phase Sampling	October 2011	Sample specific locations (surfaces and air) in the rendering plant for the surrogates

1.2 Plant Description

The rendering facility selected for this study was the Darling International, Inc., (Darling) rendering plant located at 601 SE 18th Street, Des Moines, Iowa (Figure 1). The Darling rendering plant processes “animal by-product materials for the production of tallow, grease, and high-protein meat and bone meal” [5]. Raw materials such as animal by-product materials, animal carcasses, grease, feathers, offal, and blood are collected from a variety of commercial locations including butcher shops, supermarkets, poultry processors, slaughterhouses, farms, ranches, and feedlots. From these raw materials, the Darling rendering plant produces products that are used in livestock and poultry feed, soap, inedible tallow, and grease.

Figure 1. Photograph of Rendering Plant Test Site

The Darling rendering plant uses a dry rendering process to produce animal feed ingredients, biodiesel feedstocks, and other non-food products [5] from animal carcasses and food animal slaughter offal. The process involves the use of steam to cook the raw material and accomplish separation of the fat (Figure 2). Dry rendering is a batch or continuous process that dehydrates raw material to release fat. Following dehydration in batch or continuous cookers, the melted fat and protein solids are separated as final products.

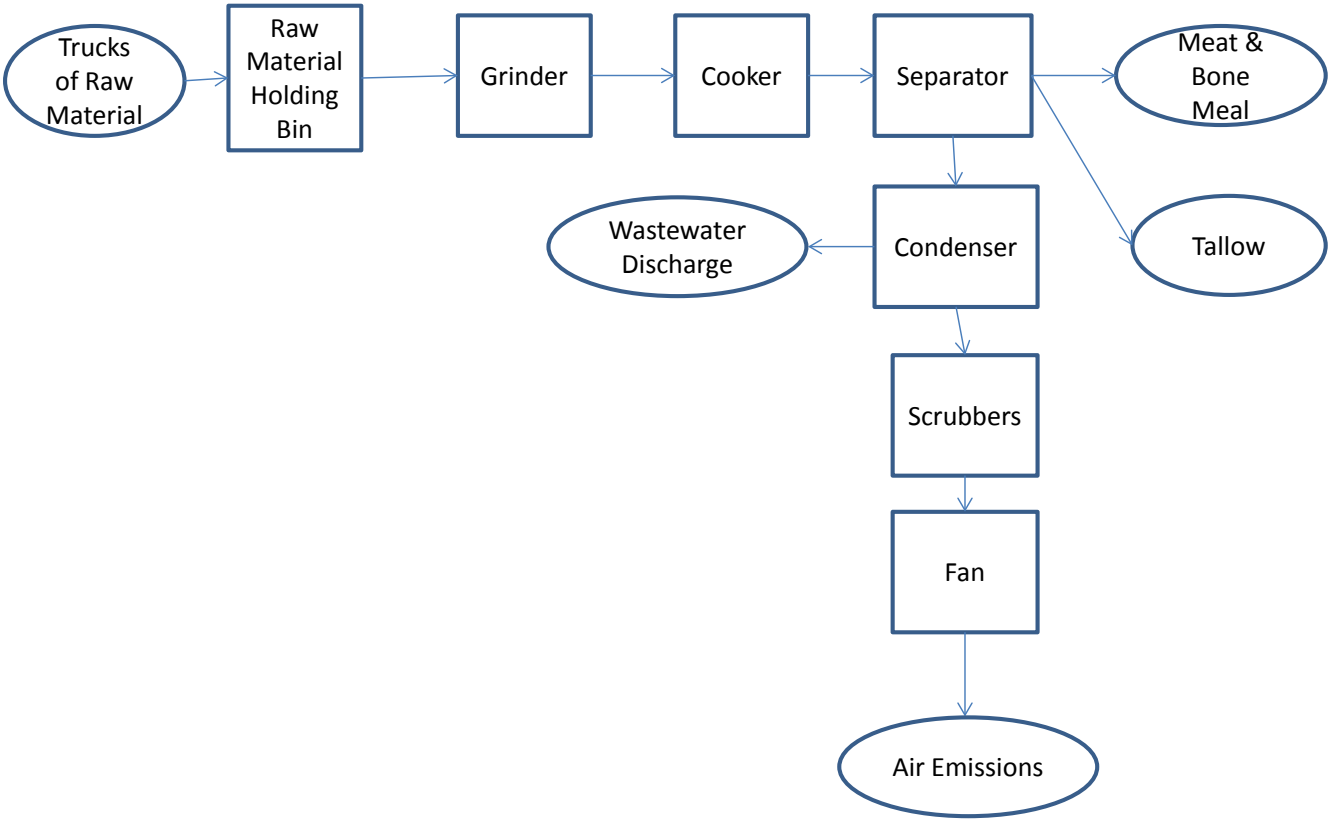

Figure 2. Conceptual Diagram of Dry Rendering Process

2. EXPERIMENTAL PROCEDURES: SELECTION OF SURROGATE FOR CLEANING/INOCULATION FIELD TEST

2.1 Initial Site Visit and Preliminary Scoping Samples

2.1.1 Purpose and Description

On January 6, 2010, the Test Team toured the Darling International plant. Sampling was initially not planned at the plant tour, and wetting solution was not available, but opportunistic swab samples were acquired from plant surfaces to provide background information that could be used for the Initial Plant Sampling effort. During the visit, a total of six opportunistic swab samples were collected by EPA personnel. Each swab sample was collected from a 10 centimeter (cm) by 10 cm area with a dry, unsterilized swab and placed in a nonsterile resealable plastic bag. Samples were logged on a facility map, pictures were taken of the sample locations, and the time/date of sampling was recorded. The swabs were stored in the swab container and placed in a Ziploc® bag. Test team members from Research Triangle Park, NC (RTP), retained custody and carried the samples back to RTP with them. All samples were streak-plated at the EPA Office of Research and Development's (ORD's) laboratory in RTP onto two Tryptic Soy Agar (TSA) plates. One plate for each sample was incubated at 35 degrees Celsius (°C) and the other at 55 °C for 24 hours (hr) to obtain an identification of bacteria that were present.

2.1.2 Results

Results indicated no growth (zero colony forming units [CFU]) on all but two of the samples. The two samples with growth were collected from the auger leaving the receiving floor and from the carcass entry door (both incubated at 35 and 55 °C) near where the trucks of raw material deposit their load prior to the carcasses being placed in the holding bin (see Figure 2).

2.2 Initial Plant Sampling

2.2.1 Purpose and Description

Because of the anticipation of seeing a rich collection of bacterial flora in all of the rendering plant samples, prior to performing tests on effectiveness of plant cleanup activities prior to and after inoculation, it was necessary to identify an appropriate surrogate organism or material to use for the field testing. The surrogate(s) to be used should have the following characteristics:

- Not be present in the background flora of the plant;
- Be able to be identified in the matrices of interest in the rendering plant (dead animals, meat and bone meal, tallow, wastewater); and
- Be able to be separated analytically from the probable high levels of background bacterial flora in the rendering plant samples.

On June 15, 2010, after Test Team personnel gave plant personnel necessary sampling supplies, materials, and directions on how to take the samples, rendering plant personnel collected environmental surface samples at the Darling rendering plant. Figure 3 illustrates the sample locations. The samples were collected using sterile swabs moistened with either Amies (Liquid Amies, Single Swab, BD Diagnostics #220093; purchased from VWR Scientific, Suwannee, GA, USA – VWR #90001-036) or Stuart's (Liquid Stuart, Single Swab, BD Diagnostics #220099; purchased from VWR Scientific, Suwannee, GA – VWR #90001-040) transport media. Odd-numbered samples were collected using Amies transfer media, and even-numbered samples were collected using Stuart's transfer media to evaluate the efficacy of each medium. Plant personnel shipped the samples on ice to Clemson University for analysis

(See Appendix A for the complete report from Clemson). Table 2 summarizes the samples collected during the Initial Plant Sampling event.

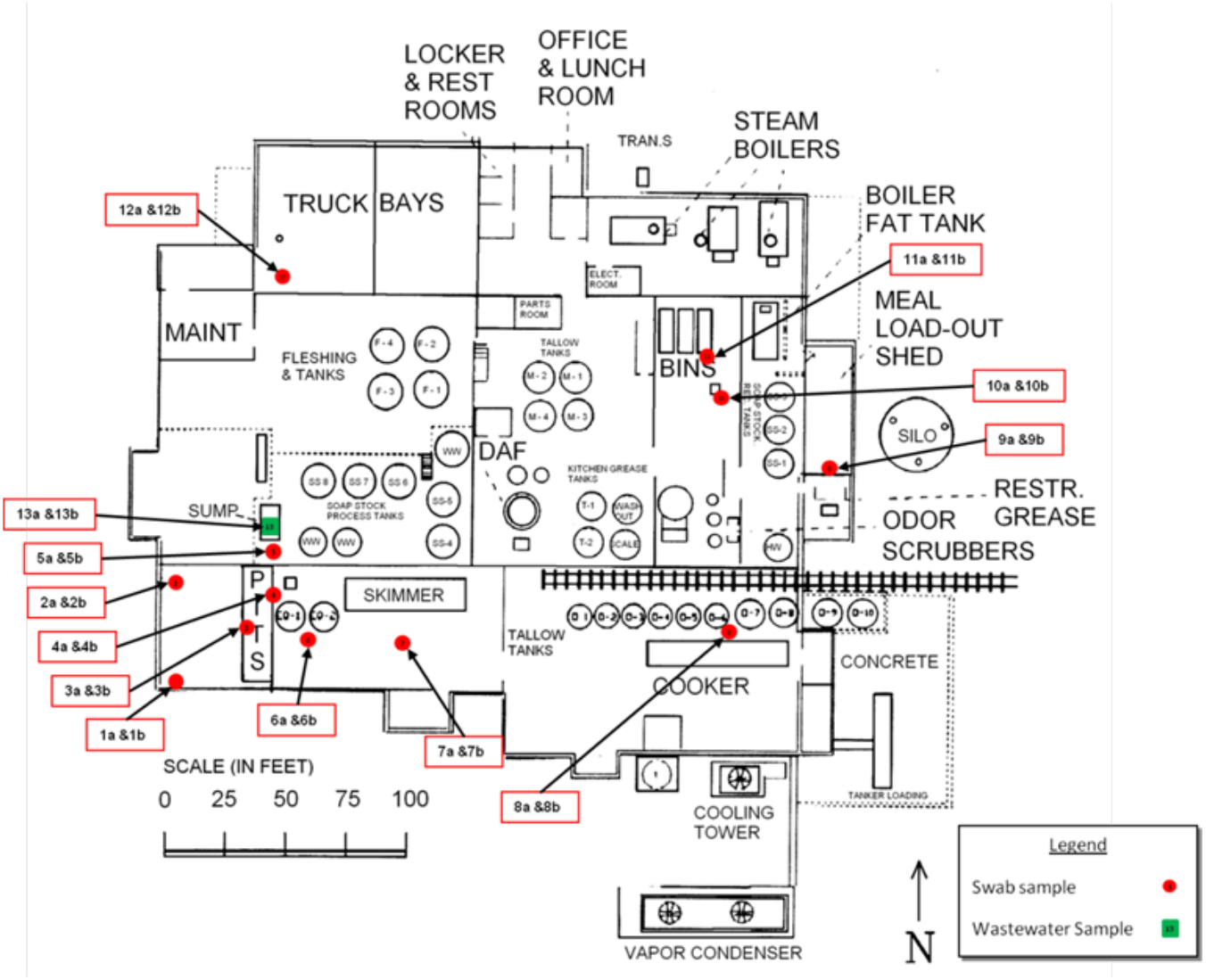

Figure 3. Sampling Map for the June 15, 2010, Initial Plant Sampling

Table 2. Samples Collected During Initial Plant Sampling Activities

Sample Number	General Location Description	Matrix	Measurement*	Total Samples
1a, 1b	Raw receiving floor area #1	Swab of surfaces	Polymerase chain reaction (PCR)/deoxyribonucleic acid (DNA) Sequencing (a) & Culture/Enumeration (b)	Facility – 24
2a, 2b	Raw receiving floor area #2	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
3a, 3b	Pit area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
4a, 4b	Pit Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
5a, 5b	Sump Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
6a, 6b	Raw Material Incline Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
7a, 7b	Raw Grinder Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
8a, 8b	Tallow Tanks/Dryer Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
9a, 9b	Load Out Screw (North End)	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
10a, 10b	Crax Grinder Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
11a, 11b	Crax Storage Bin Area	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
12a, 12b	Tailgate of Truck in Receiving Bay	Swab of nonporous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	
13a, 13b	Wastewater from Raw Pit Sump	Liquid	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	2
14a through 16b	Laboratory Blanks	Agar blank, diluent blank, and swab blank	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	6
17a, 17b	Field Blank	Swab prepared in field as a sample	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	2
18	Positive Control (PCR / DNA sequencing)	Pure culture of *G. stearothermophilus*	PCR/Sequencing	1
19	Positive Control (swab spike)	Swab spiked with 1E4 CFU *G. stearothermophilus*	Culture/Enumeration	1
20	Positive Control (extraction buffer spike)	Extraction buffer spiked with 1E4 CFU *G. stearothermophilus*	Culture/Enumeration	1

* See Appendix A for details on analytical procedures.

The swab samples were used to inoculate Brain Heart Infusion (BHI) broth tubes. The BHI tubes were incubated overnight at 35 °C and 55 °C. The 55 °C pre-enrichment broth cultures were streaked for isolation on BHI agar (BHIA) and incubated overnight at 55 °C. Growth was detected at 55 °C on 28 of the 32 collected samples. The 35 °C pre-enrichment broth cultures

were streaked for isolation on BHIA and incubated overnight at 35 °C. At 35 °C, copious growth was detected on all 32 samples. From the streak plates incubated at 55 °C, 32 pure cultures were isolated on BHIA slants which were incubated at 55 °C. Five additional 55 °C plates contained prolific spreader organisms which were not isolated during this study. PCR analysis was conducted on the 32 isolated cultures to amplify the 16S ribosomal ribonucleic acid (rRNA) gene from the bacterial isolates. Bacterial identity was selected from the top 25 BLASTn database results with maximum identity greater than 90%. Gram reaction and morphological characteristics were utilized to confirm the identity of bacterial isolates.

2.2.2 Results

In the initial experiment, only 14 isolates were successfully amplified and submitted for sequencing. Results from this set of isolates are shown in Table 3.

Table 3. Results from First Isolation Attempt

1. *B. licheniformis* 90%	6. No result returned	11. *Tepidiphilus* sp. or *Petrobacter* sp. 83%
2. *B. licheniformis* 81%	7. No result returned	12. *Tepidiphilus margaritifer* 99%
3. *B. licheniformis* 88%	8. No result returned	13. *Aneurinibacillus thermoaerophilus* 91%
4. No result returned	9. No result returned	
5. No result returned	10. No result returned	
14. *Aneurinibacillus thermoaerophilus* 91%		

In the second isolation attempt, 72 isolates were obtained. Many of these isolates were deemed likely duplicates based on Gram stain and morphology. After amplifying, these 72 isolates were submitted with four positive controls in duplicate (eight in total). The positive controls were American Type Culture Collection (ATCC) 7953 *Geobacillus stearothermophilus*, ATCC 12980 *G. stearothermophilus*, ATCC 12978 *G. stearothermophilus*, and SPORTROL* Spore Suspensions, NAMSA (VWR Scientific Products, Inc., # 19872-024). Results from this set of isolates are shown in Table 4.

Bacterial identification results using PCR and amplicon sequencing indicated lack of sensitivity of the procedure to identification of *G. stearothermophilus*. Only 37.5% of the positive controls were successfully identified as *G. stearothermophilus* by the procedure. Results of this study as well as a subsequent literature review indicated that further work on *G. stearothermophilus* may require construction of *Geobacillus* genus-specific (GEOBAC) primers specific to the *Geobacillus* genus based on Internal Transcribed Spacer (ITS) sequences [6].

2.3 Initial Surrogate Evaluation

2.3.1 Purpose and Description

A second more systematic sampling effort was then undertaken, using the information gathered during the Initial Plant Sampling Event, in an effort to focus on an appropriate surrogate organism.

On October 18-20th, 2010, 26 samples were collected (twenty-four swab and two wastewater samples) from 13 areas of the Darling plant. Two swab samples were collected from adjacent areas at 12 sample locations that included the receiving floor, hard surfaces, grinders, and crax area. One of the swabs was used for community characterization (PCR/DNA sequencing), and the other swab was used for bacterial enumeration via dilution plating. The two wastewater samples were collected from the wastewater (from scrubber discharge) collection sump near the equalizing tanks. Four positive controls in duplicate (eight total) were also sent to the laboratory for analysis. See Section 3.5 for a description of the analytical procedures that were used.

Table 4. Results from Second Isolation Attempt

1. No result returned	94%	54. No result returned
2. *G. stearothermophilus* 77%	26. *Bacillus sp.* 97%	55. No result returned
3. No result returned	27. No result returned	56. No result returned
4. No result returned	28. No result returned	57. No result returned
5. *Geobacillus* sp. 96% or *G. pallidus* 94%	29. No result returned	58. *B. licheniformis* 94%
6. *B. coagulans* 97%	30. No result returned	59. *Klebsiella pneumonia* 93%
7. *No result returned	31. *Klebsiella pneumonia* 93%	60. No result returned
8. **G. stearothermophilus* 92%	32. No result returned	61. No result returned
9. †No result returned	33. No result returned	62. No result returned
10. †No result returned	34. No result returned	63. *B. licheniformis* 77%
11. §*G. stearothermophilus* 97%	35. No result returned	64. No result returned
12. §No result returned	36. No result returned	65. No result returned
13. ‡No result returned	37. No result returned	66. No result returned
14. ‡*G. stearothermophilus* 97%	38. No result returned	67. No result returned
15. *Klebsiella* sp. 99%	39. No result returned	68. No result returned
16. No result returned	40. No result returned	69. No result returned
17. *B. coagulans* 97%	41. No result returned	70. No result returned
18. *G. pallidus* 99%	42. No result returned	71. No result returned
19. Klebsiella sp 97%	43. No result returned	72. *B. licheniformis* 96%
20. No result returned	44. No result returned	73. *B. thermoamylovorans* 97%
21. No result returned	45. *Aneurinibacillus thermoaerophilus* 96%	74. *Brevibacillus* sp 86%
22. No result returned	46. No result returned	75. *Brevibacillus* 84%
23. No result returned	47. No result returned	76. No result returned
24. *Tepidiphilus* sp. or *Petrobacter* sp. 94%	48. No result returned	77. *B. thermoamylovorans* 94%
25. *B. thermoamylovorans*	49. No result returned	78. *Bacillus* sp. 90%
	50. No result returned	79. *B. licheniformis* 95%
	51. No result returned	80. No result returned
	52. No result returned	
	53. No result returned	

* Positive Control = ATCC 7953 *G. stearothermophilus*
†Positive Control = ATCC 12980 *G. stearothermophilus*
§Positive Control = ATCC 12978 *G. stearothermophilus*
‡Positive Control = SPORTROL* Spore Suspensions, NAMSA

2.3.2 Results

The results of the sampling activities are summarized in Table 5. Several *Bacillus* species were identified as well as some potential positive identifications of *Geobacillus* species. *Bg* was not identified in the background samples for these tests.

Because thermophilic bacterial enumeration results revealed wide variability between duplicates, the experimental procedure on swab samples using BHI and both standard phosphate (PO_4)/magnesium chloride ($MgCl_2$) and lecithin buffer was repeated twice. The problems with the variability of the results and the lack of the ability to identify the preferred surrogate organism (*G. stearothermophilus*) successfully using PCR resulted in re-evaluation of the surrogate to use for the Inoculation and Cleaning tests. Table 6, below, summarizes the various species identified during both the June and October sampling events.

Table 5. Summary of the October 18-20, 2010, Environmental Surface Swab Sampling Results from Darling International, Inc., Rendering Plant

Sample Number	Sample Location	Predominant Bacteria Identity (>90% Identity Match, unless stated)
1A	Raw receiving door jamb	*B. licheniformis*
1B	Raw receiving door jamb	*B. licheniformis*
2	Raw receiving door jamb	indeterminate
3	Raw receiving door jamb	*Brevibacillus thermoruber*
4	Raw receiving door jamb	*Dictyostelium discoideum* (soil-living amoeba; only 72% match)
5	Concrete drive outside raw receiving bay	*Geobacillus* spp. (*G. pallidus* = top match 98%)
6	Concrete drive outside raw receiving bay	*Geobacillus* spp. (*G. pallidus* = top match 84%)
7A	Concrete drive outside raw receiving bay	*B. aestuarii*
8	Concrete drive outside raw receiving bay	*G. thermodenitrificans*
9	Concrete drive outside raw receiving bay	*B. aestuarii*
10A	Concrete drive outside raw receiving bay	*Brevibacillus brevis*
10B	Concrete drive outside raw receiving bay	*B. aestuarii*
12A	Raw receiving floor	*Ureibacillus thermosphaericus*
12B	Raw receiving floor	*Petrobacter* spp.
13	Raw receiving floor	*Aneurinibacillus thermoaerophilus*
14	Raw receiving floor	*Geobacillus* spp.
15A	Raw receiving floor	*Geobacillus* spp. (*G. toebii* = top match 87%)
15B	Raw receiving floor	*Tepidiphilus margaritifer*
16	Raw receiving floor	*Aneurinibacillus thermoaerophilus*
17	Back of pit – dried material	*G. pallidus*
19A	Front face of pit – mixed material	*B. coagulans*
19B	Front face of pit – mixed material	indeterminate
20	Front face of pit – mixed material	*B. thermoamylovorans*
21	Front face of pit – dried material	*B. coagulans*
22	Front face of pit – dried material	*B. coagulans*
23	Raw material incline auger – dried material	Indeterminate
24	Raw material incline auger – dried material	*B. licheniformis*
25	Top cover – raw grinder	*G. thermodenitrificans*
27	Crax grinder housing	*B. aestuarii*
28	Crax grinder housing	*Aneurinibacillus thermoaerophilus*

Table 6. Species Identified during June and October 2010 Sampling Events

Sample Location	Result**
13	*Aneurinibacillus thermoaerophilus*
16	*Aneurinibacillus thermoaerophilus*
28	*Aneurinibacillus thermoaerophilus*
13	*Aneurinibacillus thermoaerophilus* 91%
14	*Aneurinibacillus thermoaerophilus* 91%
45	*Aneurinibacillus thermoaerophilus* 96%
7A	*B. aestuarii*
9	*B. aestuarii*
10B	*B. aestuarii*
27	*B. aestuarii*
19A	*B. coagulans*
21	*B. coagulans*
22	*B. coagulans*
6	*B. coagulans* 97%
17	*B. coagulans* 97%
1A	*B. licheniformis*
1B	*B. licheniformis*
24	*B. licheniformis*
63	*B. licheniformis* 77%
2	*B. licheniformis* 81%
3	*B. licheniformis* 88%
1	*B. licheniformis* 90%
58	*B. licheniformis* 94%
79	*B. licheniformis* 95%
72	*B. licheniformis* 96%
78	*Bacillus sp.* 90%
26	*Bacillus sp.* 97%
20	*B. thermoamylovorans*
25	*B. thermoamylovorans* 94%
77	*B. thermoamylovorans* 94%
73	*B. thermoamylovorans* 97%
75	*Brevibacillus* 84%
10A	*Brevibacillus brevis*
74	*Brevibacillus sp* 86%
3	*Brevibacillus thermoruber*
4	*Dictyostelium discoideum* (soil-living amoeba; only 72% match)
17	*G. pallidus*
18	*G. pallidus* 99%
5	*Geobacillus sp.* 96% or *G. pallidus* 94%
14	*Geobacillus spp.*
6	*Geobacillus spp.* (*G. pallidus* = top match 84%)
5	*Geobacillus spp.* (*G. pallidus* = top match 98%)
15A	*Geobacillus spp.* (*G. toebii* = top match 87%)
2	*G. stearothermophilus* 77%
8*	*G. stearothermophilus* 92%
11§	*G. stearothermophilus* 97%
14‡	*G. stearothermophilus* 97%
8	*G. thermodenitrificans*
25	*G. thermodenitrificans*
2	indeterminate
19B	indeterminate
23	indeterminate
31	*Klebsiella pneumonia* 93%
59	*Klebsiella pneumonia* 93%
19	*Klebsiella sp* 97%
15	*Klebsiella sp.* 99%
12B	*Petrobacter spp.*
15B	*Tepidiphilus margaritifer*
12	*Tepidiphilus margaritifer* 99%
11	*Tepidiphilus sp. or Petrobacter sp.* 83%
24	*Tepidiphilus sp. or Petrobacter sp.* 94%
12A	*Ureibacillus thermosphaericus*

Notes:

* Positive Control = ATCC 7953 *G. stearothermophilus*
† Positive Control = ATCC 12980 *G. stearothermophilus*
§ Positive Control = ATCC 12978 *G. stearothermophilus*
‡ Positive Control = SPORTROL* Spore Suspensions, NAMSA

Identified in June sampling event
Identified in Oct sampling event; 14 isolates round
Identified in Oct sampling event; 72 duplicates round

** Percentages reflect statistical confidence in identification of a specific organism

These results suggested the following considerations regarding selection of a biological surrogate for the cleaning/inoculation study:

- *Bg* spores do not appear to exist in the background flora at this particular rendering plant; and
- *G. stearothermophilus* spores, although thermophilic in nature and likely to simplify analyses by allowing incubation at temperatures that would kill much of the background flora, cannot be detected reliably using PCR in the positive controls, let alone when mixed with other bacterial species.

2.4 Preliminary Selection of Surrogates

As previously mentioned, selection of an appropriate surrogate with the following characteristics for plant inoculation tests was desired:

- Not to be present in the background flora of the plant;
- Able to be identified in the matrices of interest in the rendering plant (dead animals, meat and bone meal, tallow, wastewater); and
- Able to be separated analytically from the probable high levels of background bacterial flora in the rendering plant samples.

The Initial Plant Sampling Event did not observe any of the spore-forming *Bacillus* species commonly used as surrogate organisms for decontamination studies, particularly *G. stearothermophilus* and *Bacillus atrophaeus* (also known as *Bacillus globigii*, or *Bg*). A thermophilic bacterium like *G. stearothermophilus* would likely be the best surrogate because the high incubation temperature during culturing (55 °C) would preclude the growth of many of the background microorganisms that could confound analysis. However, the Initial Plant Sampling also observed that *G. stearothermophilus* was not able to be identified consistently and positively using PCR even from the positive controls. This lack of *Geobacillus*-specific primers is a significant obstacle to using *G. stearothermophilus* as a surrogate in a situation where high concentrations of background flora would require positive identification using PCR. *G. stearothermophilus* was, therefore, abandoned as a potential surrogate, and *Bg* was selected as the biological surrogate to be used for the field tests.

Because of the uncertainties associated with using a nonthermophilic surrogate organism in the field tests, a nonbiological surrogate was also chosen to use in the inoculum. This nonbiological surrogate needed to be biodegradable and compatible with rendering plant products. To maximize the ability to detect the surrogate utilized, food-grade Phosphorex, Inc. DegraFluorex™ PLGA fluorescent microspheres (catalog #LGFG1000, lot # 101028-187) were selected for inclusion in the inoculum as a second surrogate with the spore-forming bacterium *Bg*.

2.5 Rendering Matrix Challenge Testing

2.5.1 Purpose and Description

Now that the proposed surrogates had been identified, verification that they could indeed be recovered analytically from the likely environmental matrices found in a rendering plant was desired.

Bg and PLGA were used to spike protein-based stock (i.e., suet) (1 gram (g) each), grease (1 milliliter (mL) each), and deionized (DI) water (1 mL each). These media were spiked with *Bg* spores at a concentration of 1E8 CFU/sample (0.1 mL of a 1E9 CFU/mL culture). Separate portions of meat and grease and DI water were spiked with PLGA microspheres [1 micrometer (µm); green color; Ex/Em (nanometers [nm]) 460] at approximately 1E6 beads/g or mL (0.1 mL

of a 3 microgram (µg)/mL solution was added to 1 g or 1 mL of meat or grease, respectively) after nucleic acid extraction. The purpose of these samples was to assess the ability of the laboratory to identify *Bg* through PCR and measure *Bg* and PLGA from matrices simulating the matrices found at a meat-rendering facility.

Bg DNA was detected using a real-time polymerase chain reaction (RT-PCR) assay specific for the sequence encoding the Replication Termination Protein (rtp) present on the *Bg* chromosome, and PLGA microspheres were detected by direct microscopic count. The *Bg* rtp RT-PCR assay was established using a standard curve prepared from *Bg* genomic DNA and tested using spiked samples. Direct microscopic counts of PLGA were performed using disposable hemacytometers (INCYTO, part number: DHC-NO1-5) and a Zeiss epifluorescent microscope (Carl Zeiss International, Jena, Germany).

2.5.2 Results

Preliminary tests were conducted in meat, grease, and water spiked with *Bg* spores and PLGA microspheres. Detection was carried out via RT-PCR and fluorescent microscopy. The laboratory provided their results and recommendations, which included the use of inocula >1E8 CFU/g or mL, and extraction using a commercial kit or dilution to overcome inhibition. *Bg* DNA was detected in water samples but not in meat or grease samples when analyzed directly. After extraction of nucleic acids using a commercial kit, *Bg* DNA was detected in all three matrices. Recovery of *Bg* DNA signatures was detected in 6-7% of water samples and in less than 1% of meat and grease samples.

PLGA microspheres were detected within the quantification range when visualized in water or meat samples using microscopy. However, autofluorescence from the grease at the same wavelength as the PLGA particles inhibited efficient detection and counting of PLGA microspheres in grease samples.

2.5.3 Significance of Challenge Test Results

The results of the challenge tests showed that *Bg* DNA and PLGA microspheres could be detected in water, but recovery percentages were not high. *Bg* DNA recoveries were <10% in water and <1% in meat and grease samples. Additionally, *Bg rtp* was not detected in direct analysis of meat or grease samples. Proteases and nucleases present in the meat and grease matrices as well as other PCR-inhibitors may have prevented direct detection of target DNA. Based on these challenge test results, the Test Team determined that to ensure efficient distribution of the spike within the sample matrix and sufficient recovery of target signatures, spikes should be prepared to contain approximately 1E8 CFU and 1E8 beads per g or mL of crude protein.

PCR inhibition can be overcome by dilution or by extracting the nucleic acid samples from the sample matrix. Also, extraction using a simple DNA purification kit may also result in detectable signatures in the meat and grease samples, but at levels lower than the expected concentration (recovery was less than 1%). Sample dilution might be a better alternative for these sample matrices or a more desirable solution for the end users, but testing would be necessary to determine the optimal dilution to overcome PCR inhibition without significant loss of target DNA. However, dilution comes at the expense of sensitivity; it is not clear whether a different/additional purification step would be better or if diluting the inhibitor is better. An internal positive control (IPC) kit designed to test for the presence of inhibitors in PCR samples by analysis of an exogenous target DNA could also possibly be used to test neat and diluted samples prior to analysis to determine the optimal conditions for *Bg rtp* detection. The Test Team decided that samples should undergo an extraction procedure, either using a commercially-available kit for purification of DNA or other standardized method, prior to analysis

by RT-PCR.

In addition, grease samples were found not to be amenable to direct microscopic observation, and PLGA microspheres are not distinguishable from the matrix due to background autofluorescence. PLGA microspheres may possibly be washed or extracted from the grease, or sample dilution could possibly overcome the interference, but further experimentation would be required to develop a feasible method for visualizing PLGA microspheres from the grease matrix. In addition, *Bg* cells may be visible in grease samples under phase contrast or in the presence of an appropriate stain, but further research would be necessary to develop an appropriate visualization method.

2.6 Final Surrogate Selection

Based on the results of the sampling and methods development work done, an ideal surrogate for use in the field test did not appear to exist. Tradeoffs must be taken into account and a balance struck to pick the best available surrogate given the amount of information that was currently available.

Based on the results of the laboratory challenge samples, initial suggestions proposed that the inoculum for the study would consist of an aqueous mixture containing 1E11 CFU of *Bg* spores and 1.47E9 beads of PLGA microspheres dissolved in 1 gallon (gal) of distilled water, sprayed over each truckload of raw material, to be sprayed evenly over the load (approximately 20 tons) in each truck that arrived on site during the inoculation portion of the study (note – the proposed inoculum would be only on the outside of the materials in the truck and would not be evenly distributed within the 20 ton load). A surfactant would be added to the mixture to reduce clumping. The Test Team also received information that 1E11 CFU of *Bg* spores tend to clump together and produce a much lower level of contamination [7]. By adding a surfactant to the mixture, 1E9 *Bg* spores per mL could be utilized more effectively in the study. Clumping would be reduced, and the estimated level of contamination would be greater than 1E9 of *Bg* per truckload. In addition, significant cost savings could be realized. Thus, a surfactant, “Fluid D” (see Appendix E), was added to the final mixture of 1E9 CFU of *Bg* spores and 1.47E9 beads of PLGA/gal.

3. EXPERIMENTAL PROCEDURES: CLEANING/INOCULATION FIELD TEST

3.1 Test Design/Planned Approach

The Cleaning/Inoculation Testing included the following elements in chronological order:

- Pre-clean the plant to remove the bulk of any built-up organic material that had accumulated on various plant surfaces;
- Perform background sampling of surfaces and air at pre-identified locations within the plant and in outside perimeter locations for *Bg* spores and PLGA microspheres;
- Inoculate each truckload of raw material (animal carcasses, offal) entering the plant over the course of an eight-hour day with the solution containing *Bg* spores, PLGA microspheres, and surfactant; perform air sampling for components of the inoculum during the inoculation part of the study;
- Perform post-inoculation sampling of surfaces and air at pre-identified locations within the plant and in outside perimeter locations for the same target analytes;
- Clean pre-determined areas of the plant with existing plant cleaning procedures, using hot water and steam; and
- Perform post-cleaning sampling of surfaces and air at pre-identified locations within the plant and in outside perimeter locations.

Table 7 lists the detailed timeline of events for the Cleaning/Inoculation Tests, including the number of samples collected, types of samples collected, and other notations regarding the procedures that were used.

A planned approach was developed that identified 124 sample locations throughout the rendering plant, including the process room, grinders, and outside the cooker (Figures 4 and 5). Thirty-four air sample locations were selected randomly inside and outside the plant while 90 wipe sample locations were pre-determined. Outside air samplers were to be positioned on all sides of the plant, but the majority of the samples were to be collected downwind of plant operations. Inside air sampler locations were to be concentrated in high dust areas or areas where crushing and grinding could aerosolize the surrogates from the rendering process or from fomites.

Unlike the Preliminary Scoping Samples that utilized swabs, four wipes were collected from each of the 90 surface sampling locations. One wipe (designated as A) was collected for community characterization by PCR, one wipe was collected for enumeration (designated as B), and the third wipe was collected for PLGA identification (designated as C). A fourth sample was collected and stored for archival purposes (designated as D). Wipe samples were chosen over swabs because the wipes provided a slightly larger surface area and wipes are routinely used by EPA to sample surfaces for biological agents. In addition, swabs were not an optimal sampling medium for a rendering environment; the tip of the swab could be impacted by a single large particle from the rendering process, and the characteristics of the material buildup on the surfaces in the rendering plant made it difficult to establish the sample area from samples utilizing swabs.

Table 7. Timeline of Events for the Cleaning/Inoculation Portion of Study

Day*	Day of Week	Time	Primary Task	Additional Task	Samples				Notes/Assumptions
					Wipe	Wipe Blanks	Air	Air Blanks	
Sept. 24-25	Sat & Sun	Work Shift	Pre-Cleaning (weekend 1)						Weekend 1: Cleaning conducted by plant (no oversight)
Oct. 1-2	Sat & Sun	Work Shift	Pre-Cleaning (weekend 2)						Weekend 2: Cleaning conducted by plant (no oversight)
Oct. 8-9	Sat & Sun	Work Shift	Pre-Cleaning (weekend 3)	Oversight / Documentation, Finalize Sample locations					Weekend 3: Cleaning conducted by plant (Test Team oversight).
Oct. 17	Mon.	Work Shift	Background Samples	Scoping and Prep, Documentation, Package and Ship samples	4	1	7	1	Collect background samples throughout entire facility.
Oct. 20-21	Thurs., Fri.	As trucks are available	Inoculate loads of carcasses	Documentation			5		Inoculate carcasses as loads arrive either off-site or in a containment area to prevent spreading. 1) Spray the carcasses down; 2) Spike carcasses in each load with the surrogate. 3) Continue inoculating for one 8-hr shift.
Oct. 21	Fri.	Work Shift (8 hours)	Process Contaminated Carcass	Sampling, Documentation					Inoculated material will be processed for eight hours.
Oct. 21	Fri.	Work Shift (8 hours)	Stage 1 - Process Sampling	Documentation, Package and Ship samples	8	2	4	1	Sampling during processing of inoculated material. Eight hr air samples will be initiated in the process area and throughout the building. Surface wipe samples will be taken every two hr from grinder feed
Oct. 21	Fri.	Immediately afterward	Post Inoculation Sampling	Scoping and Prep, Documentation, Package & Ship samples	22	5	6	1	Immediately after all inoculated carcasses have been processed, Test Team will collect samples throughout whole facility.
Oct. 21	Fri.	After Post Dispersion Sampling	Process Clean Carcasses	Documentation					Plant will process clean material for eight hr
Oct. 21	Fri.	After process runs for 2 hr	Stage 2 - Process Sampling	Documentation	8	2			Collect surface wipe samples from the grinder. Surface wipe samples will be taken randomly every two hours from grinder feed
Oct. 22-23	Sat & Sun	All day	Plant Cleaning	Documentation					Cleaning conducted by the plant (Test Team oversight)
Oct. 23	Sun.	After cleaning is complete	Post Cleaning Sampling	Documentation, Package and ship samples	40	8	13	3	Samples collected throughout whole facility
				Total Samples	82	18	35	6	

* -- all dates are in 2011

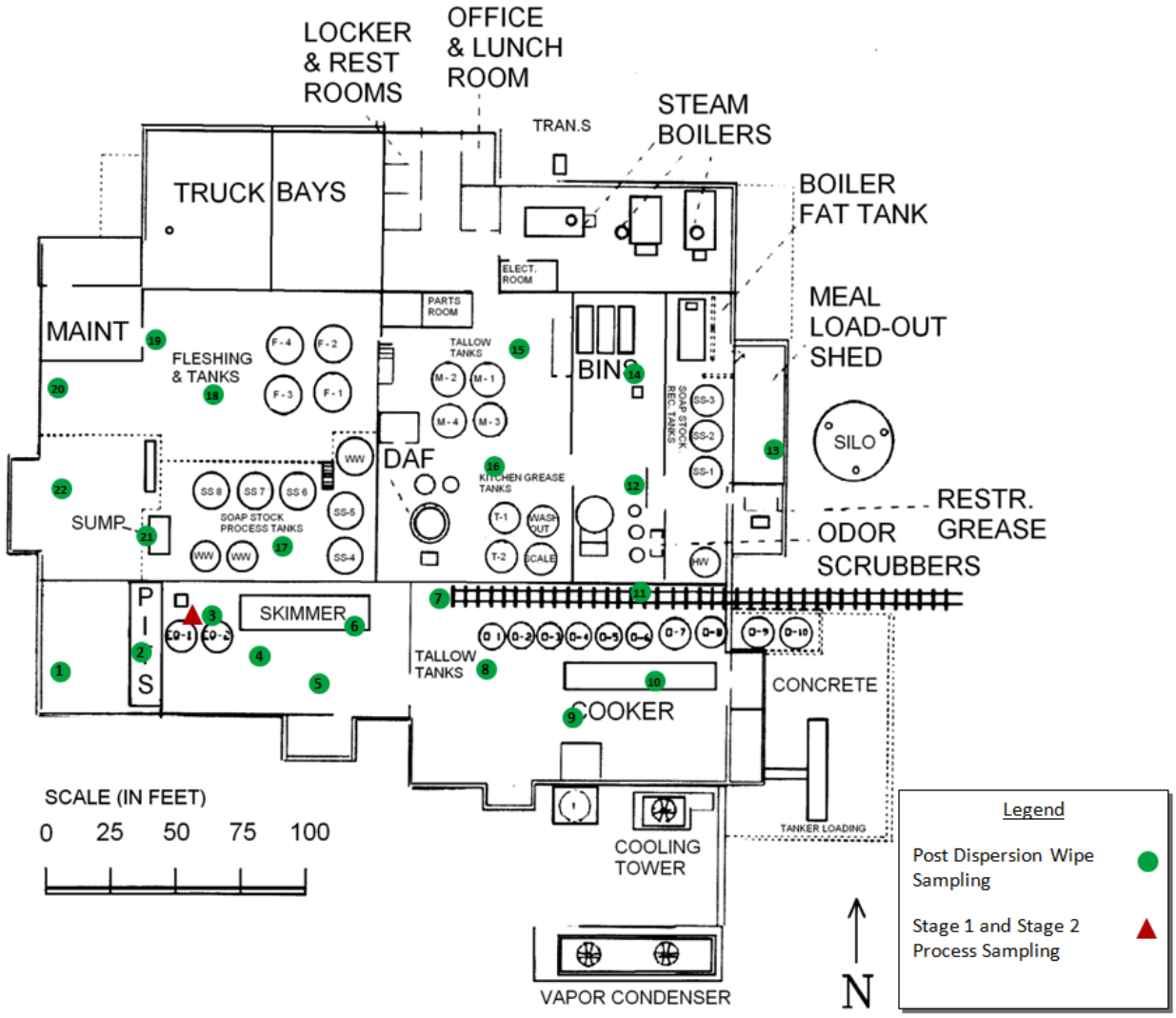

Figure 4. Post-Inoculation Sampling Locations

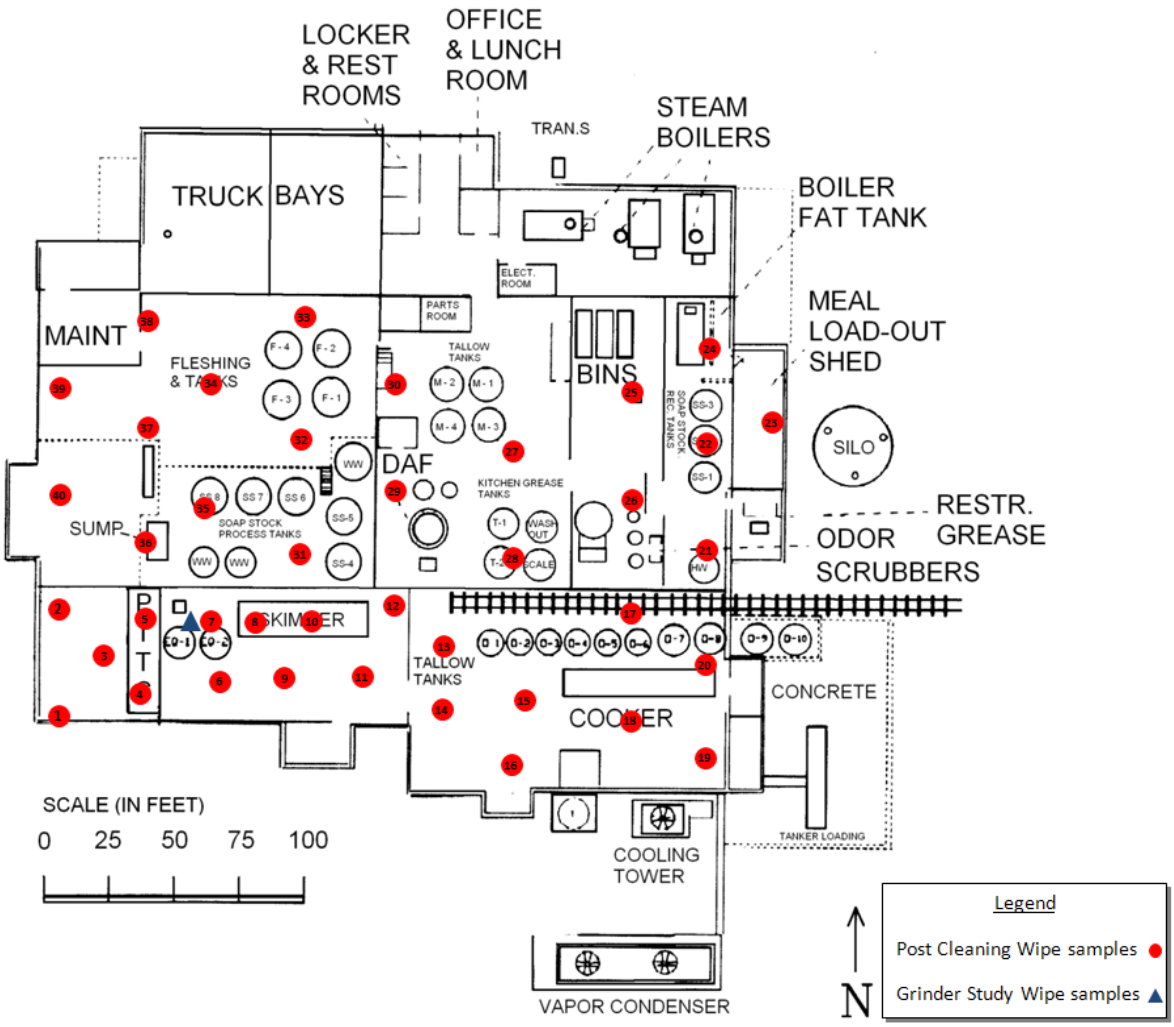

Figure 5. Post-Cleaning Study Sampling Locations

3.2 Sampling Procedures and Protocols

Sampling procedures and protocols were developed to ensure sample viability, balance the need to cover a very large plant adequately with a minimum number of representative samples, and hopefully acquire sufficient sample so that analytical results would be not be constrained by the detection limits. Twenty-eight additional Quality Assurance/Quality Control (QA/QC) samples were collected, including 27 media blanks and three inoculation solution samples. Wipes consisted of Versalon® synthetic gauze pads (Tyco Healthcare/Kendall, Versalon® All-Synthetic Sterile Sponges, 2 inches (in) x 2 in - # 8042, Mansfield, MA, USA), and the sampled areas were defined by a 10 cm x 10 cm paper template. The entire wipe was extracted. The samples were acquired at the following time intervals:

- After initial cleaning of the plant, prior to inoculation with the surrogates;
- During inoculation of carcasses (air samples only);
- After processing inoculated carcasses for eight hours; and
- After the final cleaning of the plant.

Table 7 (above) provides an outline of the planned study events. Table 8 contains a summary of samples that were collected in the background, inoculation, post-inoculation, and post-

cleaning phases. For a complete list of all sample identifications (IDs) and sample locations, see Appendix D.

Table 8. Summary of Samples Collected in the Background, Inoculation, Post-Inoculation, and Post-Cleaning Phases

Phase	Primary Task	Matrix	Measurement**	Sample Locations		QA/QC Sample Locations		
				Wipe	Air	Air Blanks	Air Spike	Wipe Blanks
Background	Pre-Cleaning (weekend 1)							
	Pre-Cleaning (weekend 2)							
	Pre-Cleaning (weekend 3)							
	Background Samples	Wipe of nonporous surfaces; MCE filter* of air	Wipe: culture and counts of CFU; PCR; PLGA Air: PLGA; biologicals	4	7	1		1
Inoculation	Inoculate loads of carcasses	Mixed Cellulose Ester (MCE) Filter of air			4	1	1	1
	Process Contaminated Carcass		Air: PLGA; biologicals					
Pre cleaning	Stage 1 - Process Sampling	Wipe of nonporous surfaces; MCE Filter of air	Wipe: Culture and counts of CFU; PCR; PLGA Air: PLGA; biologicals	8	4	1		2
	Post-Inoculation Sampling	Wipe of nonporous surfaces; MCE Filter of air	Wipe: Culture and counts of CFU; PCR; PLGA Air: PLGA; biologicals	22	6	1		5
	Process Clean Carcasses							
	Stage 2 - Process Sampling	Wipe of nonporous surfaces;	Wipe: Culture and counts of CFU; PCR; PLGA	8				2
Post cleaning	Plant Cleaning							
	Post-Cleaning Sampling	Wipe of nonporous surfaces; MCE Filter of air	Wipe: Culture and counts of CFU; PCR; PLGA Air: PLGA; biologicals	40	13	3		8
			Total Samples	82	30	7	1	19

* - Product Code 223-3-01; SKC, Eighty Four, PA, USA
** - See Section 4 and Appendix B for details on analytical methods

3.2.1 Background Sampling

Following the initial plant cleaning (see Section 3.4), a total of 11 sample locations (four surface samples and seven air samples) were sampled throughout the plant as background samples. The samples were collected prior to the release of the PLGA and *Bg* surrogates. Test personnel attempted to position the sample locations within plant areas where potential contamination was expected to be the greatest.

3.2.2 Inoculation Phase Sampling

Air samples were acquired in the following manner. Air was drawn through the filter by a pump and through a 37 mm, 0.8 µm MCE inert filter preloaded in a three-piece clear plastic cassette

(Product Code 225-3-01; SKC, Eighty Four, PA, USA). Biological agent extraction efficiencies of 99% have been determined for this type of medium [8]. The sample pumps were calibrated to operate in the 2 liters/minute (Lpm) range and collect samples over a period of time ranging from 1 to 8 hr, depending on sampling objectives and dust levels. The complete sampling equipment and procedures are below.

MCE filter samples were collected in accordance with the following procedure:

1. Pre-calibrate the sampling pump to a flow rate between 3 and 4 Lpm;
2. Don a pair of sterile or clean sampling gloves;
3. Remove the top portion of the sampling cassette;
4. Attach the filter cassette to the sampling pump inlet using Tygon® tubing;
5. Set the sample pump and filter at the sample location and connect the cassette to the sample stand;
6. Turn on the pump (sample is collected open-faced) and record start time;
7. Collect the sample for a run time of 120 minutes (min) or 2 hr;
8. Turn off the pump and record stop time;
9. Don a new pair of sterile sample gloves;
10. Disconnect the sample cassette from the sample pump;
11. Attach the top portion of the filter cassette to the sampling device;
12. Insert the end caps into the sampling cassette;
13. Label the sample;
14. Double-bag the sample;
15. Following decontamination of the samples, place the bags into a sample custody bag; and
16. Change gloves.

During the inoculation activities, a total of six air samples were collected with personal sample pumps (four test air samples and two QA/QC air samples). The purpose of these six air samples was to measure air transport of spores from the inoculation area to the plant, if there was any. All air samples were collected with SKC AirChek 2000 personal sampling pumps (SKC, Eighty Four, PA, USA) and calibrated with a Bios® DC-Lite dry cell calibrator (Bios International Corporation, Butler, NJ, USA) to 1.0 Lpm. The medium was 37-mm MCE filters in three-stage pre-loaded cassettes. Air samples were collected in four areas during the inoculation of the carcasses. Three air samples (to the east, west, and south) were collected in the area adjacent to the detarping area. A fourth sample was collected by the large door near the tipping floor.

3.2.3 Post-Inoculation Phase Sampling

During the Post-Inoculation Phase of the study, the plant processed inoculated carcasses for eight hr. Two wipe samples were collected from the grinder every two hr during the eight hr of processing (eight wipes total). Samples were collected from the grinder in the same location each time during this stage of the study. The purpose of the grinder samples was to evaluate the buildup and potential reduction of surrogate loading as the inoculated material began to be fed, continued being fed, then after the inoculated material ceased being fed. In addition, a total of four eight-hr air samples were initiated at the start of the eight-hr shift. The air samplers were distributed in the dustiest areas of the plant, including the skimmer area, the cooker area, the storage bin area, and near the plant exhaust vent. The purpose of the air samples was to characterize movement of airborne contamination within the plant. Figure 4 shows the locations of the post-inoculation samples. After the eight-hr shift was completed and all of the contaminated carcasses were processed, the plant processed uninoculated (clean) carcasses for an additional eight hr.

The plant then underwent the cleaning procedures described in Section 3.4.

3.2.4 Post-Cleaning Phase Sampling

After the cleaning had been performed by plant personnel, samples were collected from 53 locations. Thirteen air and 40 surface locations were sampled to determine the effectiveness of the cleaning. The 40 surface locations were previously shown in Figure 5. Attempts were made to acquire samples in the near vicinity of previous sample locations but not on exactly the same spot.

3.3 Inoculation of Incoming Raw Materials

During the Inoculation Phase of the study, the PLGA and *Bg* solution was evenly sprayed over the top of each truckload of raw material (i.e., animal carcasses and food animal slaughter offal) intended for processing in the rendering plant for an eight-hr shift. The PLGA and *Bg* solution was sprayed on the carcasses into and onto the load using a hand sprayer (D.B. Smith Roundup Backpack® Sprayer) containing the surrogates within a phosphate buffer solution with the added surfactant, filled to the one-gal level with distilled water. The surfactant was added to the mixture to prevent the spores from clumping together. The estimated level of contamination was ≥1E9 CFU of *Bg* and approximately 50 milligrams (mg) of PLGA (~1.47E9 spheres per mg) per truckload. The inoculum mixture was prepared immediately before its use, in one-gal batches, in the reservoir of the sprayer, and the reservoir from the sprayer was periodically shaken as spraying occurred.

Once each truck arrived at the plant, the tractor number and trailer information were documented as well as the weight of the load as measured by the plant's scales. The carcasses loaded into the truck trailer were sprayed prior to entering the facility, outside in the truck detarping area located on the south side of the plant. During one eight-hr shift, all arriving loads (approximately 16) were inoculated, and carcasses were processed. After each truck dumped its load of inoculated carcasses, the truck bed was washed out with water and dumped inside the bay prior to leaving the dumping area. Prior to leaving the site, trucks were sprayed with an amended bleach solution (1 part Clorox® bleach, 1 part white vinegar, 8 parts water) to minimize potential for cross-contamination should that truck return to the site prior to the post-cleaning sampling.

All doorways near the detarping area remained closed during the inoculations. To reduce contamination, the individual performing inoculation did not enter the plant during this stage of the study. After all of the trucks were inoculated, the person performing the inoculation was sprayed off with water prior to leaving the area, their personal protective equipment (PPE) that included Saranex® overalls and nitrile gloves was removed and disposed, and their boots were removed and left outside the plant for later use.

Table 9 lists the inoculated trucks and the load weights along with the inoculation time for the Inoculation Phase of the tests.

Table 9. Weights of Inoculated Trucks

Truck Number	Time	Truck ID	Weight (pounds)
1	0831	3638	25,780
2	0852	4193	40,300
3	0955	4578	19,840
4	1101	3996	22,100
5	1201	4987	32,240
6	1248	3842	9,540
7	1320	4901	44,060
8	1332	4564	20,800
9	1441	3553	40,780
10	1454	3982	14,740
11	1507	4015	13,340
12	1528	4062	6,860
13	1541	4189	24,480
	1622		***
14	1719	4749	15,160
15	1732	4943	37,780
Total			**367,800**

*** Positive control sample of inoculum acquired by spraying approximately 20 mL of inoculation mixture into conical tube.

3.4 Plant Cleaning

Initial observations of the Darling plant that were made on the first site visit noted that many of the surfaces of the plant had a significant bulk loading of organic material. To maximize the probability of being able to detect any surrogates that were inoculated into the raw materials entering the plant, cleaning as many of the plant surfaces as possible prior to testing was desired. Therefore, prior to any sampling as part of the cleaning/inoculation portion of the study, Darling plant workers cleaned parts of the plant over the course of several weekends to remove buildup and bulk loading of organic material from plant surfaces. Plant personnel utilized existing plant methods and external contract personnel to clean the plant. Water heated to approximately 180 to 200 degrees Fahrenheit (°F) was used to wash loose particles from plant surfaces. Plant personnel used brooms, shovels, scrapers and brushes to loosen gross contamination. Materials loosened in this manner were pushed into the pit and fed into the rendering process with the raw material. Heated water was then used to rinse the area. If existing plant water lines did not reach an area of the plant, plant personnel utilized pressure washers and a Steam Genie (Steam Genie, Inc., Compton, CA, USA) to clean those surfaces. No detergents or disinfectants were used during these steps as per routine plant procedures. Since this study was looking at the cleaning process and not the efficaciousness of disinfectants, there was not an attempt to kill the surrogate *Bg* spores. In any event, sporicidal conditions necessary to kill the *Bg* spores would have been unrealistically harsh relative to disinfectants necessary to kill the viral agents that are of most concern from an FAD standpoint. Table 10 outlines the areas (see Figure 6) to be cleaned and the cleaning methods to be utilized.

Table 10. Plant Cleaning Schedule

Date (2011)	3rd Party Cleaning Company	Cleaning by In-House Plant Staff
Sept 24-25	Raw Bay I	Raw Bay II
	Scraping lower walls, floor, receiving pits	Scraping lower walls, floor, receiving pits
	Cleaning ceiling, walls, floor	Cleaning ceiling, walls, floor
Oct 1-2	Soapstock, fleshing areas and truck bays	Duke/skimmer room
	Cleaning ceiling, walls, floor, nonelectrical equipment	Cleaning ceiling, walls, floor and nonelectrical equipment
Oct 8-9	Cooker room, meal load-out	Work tank area
	Cleaning ceiling, walls, floor, nonelectrical equipment	Cleaning ceiling, walls, floor, nonelectrical equipment
Oct 15-16	All other areas done on previous weekends	All other areas done on previous weekends
	Hot water washdown	Hot water washdown
Oct 22-23	All other areas done on previous weekends	All other areas done on previous weekends
	Hot water washdown	Hot water washdown

Following the Inoculation Phase sampling, a second cleaning was performed where plant personnel cleaned the facility. Under the limited oversight by the Test Team, plant personnel utilized existing plant methods and external contract personnel to clean the plant. Particular attention was paid to the grinder area, tipping floor, pits, the processing area, and building floors. As access allowed, plant personnel would attempt to clean any augers used in the process by utilizing typical plant cleaning procedures, which took approximately 8 hr to complete after inoculation had ceased.

3.5 Analytical Procedures and Protocols

The samples (air, wipe, and water) taken during the Cleaning/Inoculation Test were shipped overnight in a chilled container to Battelle Memorial Institute in Columbus, OH, for analysis. For more detail on the analytical report, please see Appendix B. All processing areas within the analytical laboratory, including the biological safety cabinet and incubator, were thoroughly decontaminated, and surfaces were sampled with swabs. Samples were plated onto BHIA to ensure that working areas were sterile prior to processing of rendering plant samples. An additional swab was taken and plated on BHIA on each day of sample extractions. The samples were analyzed following the procedures specified in this section.

Sample processing occurred in five batches over the course of three weeks, and positive and negative analytical controls were created for each batch as follows: a single negative control and a single positive control for each matrix type were extracted in the batch. Negative controls (Matrix Blank 1, 2, etc.) comprised a single pristine matrix, while positive controls (PCs) (Matrix PC 1, 2, etc.) comprised a single pristine matrix spiked with *Bg* DNA at 1E7 gene copies (GC)/sample and PLGA microspheres at 0.05 mg/sample. Control matrices were provided by test personnel and were identical to sample matrices. Controls were processed in tandem with the samples, and each control received treatment identical to the sample matrices.

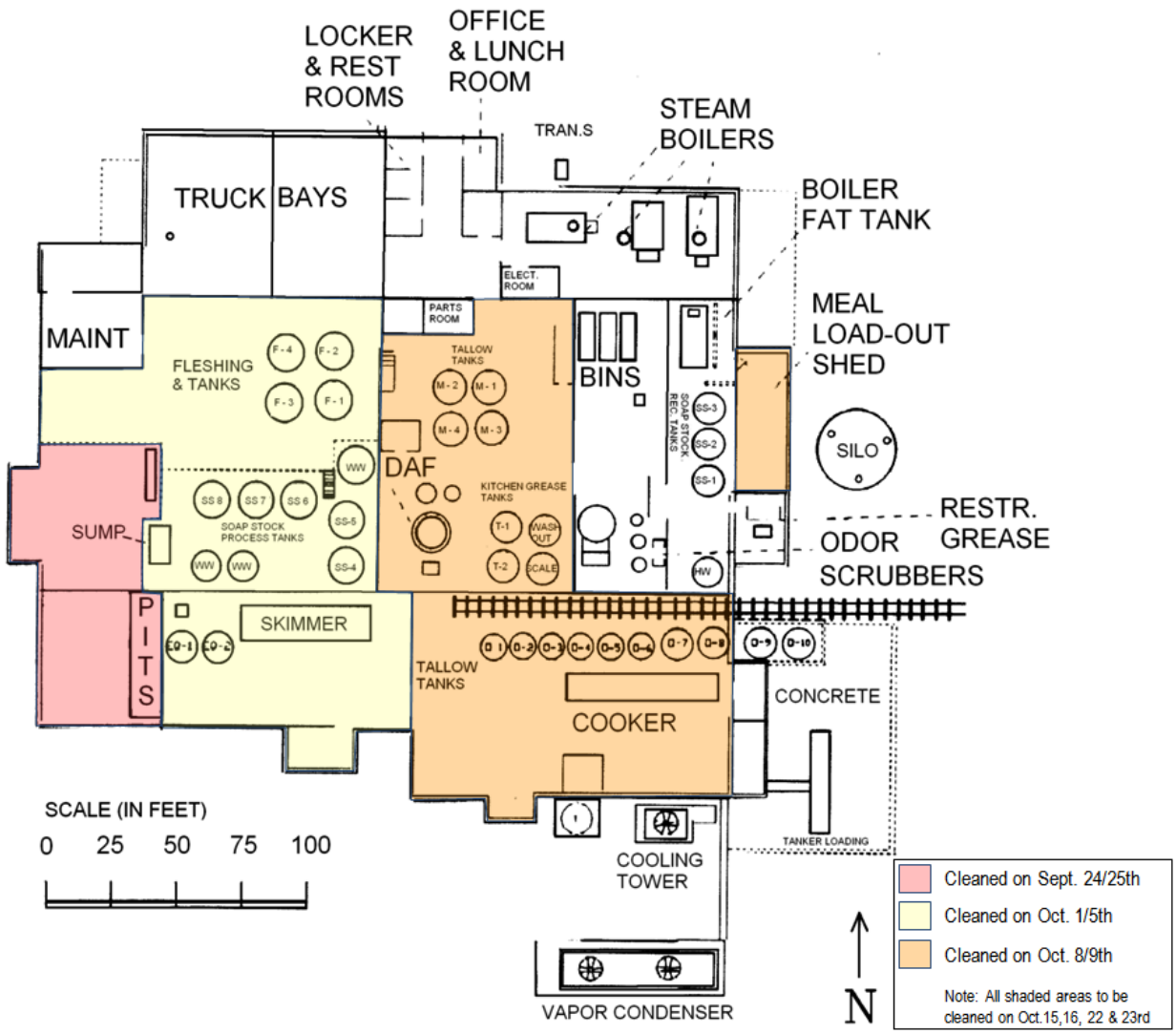

Figure 6. Areas Cleaned at the Darling Des Moines plant.

Each sample or control was extracted according to a project-specific work instruction (DWI-01-02, Work Instructions for the Extraction of Microorganisms, Nucleic Acids, and PLGA Microspheres from Environmental Samples [Appendix B]). Briefly, samples were removed from their original containers and placed into sterile 250 mL bottles, and phosphate buffered saline (PBS) was added. Each sample was mixed by vortexing for approximately 30 seconds (sec) and then incubated for 30 min at room temperature. An aliquot (1 mL) was removed to serve as the microbiology extract, and the remaining sample was extracted for nucleic acids. Microbiology extracts were plated onto BHIA and incubated at 36 ± 2 °C overnight to isolate single colonies of bacteria. The remaining microbiology extracts were stored at 4 °C until being processed further for sequence analysis. After addition of herring sperm carrier DNA (hsDNA) and 1% sodium dodecyl sulfate (SDS), samples were incubated for 30 min at 65 °C. Samples were extracted in 12 to 15 mL PBS, and 1 mL was removed for microbiological analysis. The hsDNA and 1% SDS were added to the remaining volume in the original sample extract after removal of the microbiology aliquot (i.e., membrane filter with 12 mL PBS, 1 mL removed for microanalysis, remaining volume is 11 mL). The original sample matrix was preserved in the extraction vessel for detection of PLGA microspheres by microscopic analysis, and the aqueous extract was transferred to a sterile Oakridge tube (Thermo Scientific [Nalgene], Rochester, NY,

USA). Nucleic acids were concentrated using method ABAT-V-012 (Battelle's Applied Biology and Aerosol Technology - Concentration of Nucleic Acids by Isopropanol Precipitation). In this method, nucleic acids were precipitated overnight with isopropanol, recovered by centrifugation, washed with 70% ethanol and resuspended in 1X Tris ethylenediaminetetraacetic acid (EDTA) (TE) buffer, pH 7. Spores and cells were lysed using SDS and incubated at 65 °C during the extraction process outlined in DWI-01-02 (Appendix B).

3.5.1 Bg Detection by Quantitative PCR

Samples were extracted, and nucleic acids were purified with a final volume of 200 µL. For nucleic acid analysis, duplicate 5 microliter (µL) aliquots of the 200 µL sample extracts were assayed via Quantitative PCR (qPCR) using an assay specific for the *rtp* gene of *Bg* on an ABI 7900HT platform. The limit of detection (LOD) was determined by running triplicate reactions of a standard curve prepared with *Bg* genomic DNA; the lowest concentration of DNA that is detected in the assay is considered the LOD, the lowest concentration detected in duplicate reactions is the limit of quantitation (LOQ).

To determine how many GC were present in the standard curve preparations:

1) the mass of the genome in base pairs (bp) found in the published literature was converted into pg/GCs using the conversion factor of 1.096e-21 g/bp [9];

2) genomic DNA was extracted and the amount of DNA was quantitated with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) in units of (nanograms [ng]/mL);

3) using the pg/GC factor determined in step 1, extracted genomic DNA concentration was converted to GC/mL; and

4) a standard curve was prepared in a tenfold serial dilution series.

The LOD and LOQ for this assay were determined to be 92.1 GC/5 µL. Prior to target analysis, sample extracts were tested for inhibition using the Applied Biosystems (ABI) TaqMan® Exogenous Internal Positive Control Reagents Kit (Life Technologies, Grand Island, NY, USA) according to method ABAT-V-007 (TaqMan Inhibition Analysis on the 7900HT). Neat, 1:5, and 1:10 dilutions of each sample were initially assayed. In the event extracts did not pass IPC testing at the 1:10 dilution, they were further purified using a Qiagen QIAQuick PCR Purification kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. The Qiagen-purified sample extracts were further diluted and tested by IPC analysis at Qiagen Neat (QN), Qiagen 1:5 (Q5), Qiagen 1:10 (Q10) and Qiagen 1:20 (Q20) dilutions. Sample extracts that passed IPC were analyzed for *Bg* DNA at the highest concentration passing the inhibition test according to Method ABAT-V-008 "To Prepare a 96-Well Plate for DNA Quantitation on the 7900HT" (Appendix B). The Ct (cycle threshold) value and estimated nucleic acid quantity based on the input standard curve were compiled, along with an amplification plot and a trace of fluorescent signals (multicomponent plot) for each replicate sample. The multicomponent plot was examined for each sample replicate to verify results; positive detections showed elevated signal from the reporter fluorescent molecule. Assay acceptance criteria included the following:

- Valid standard curve with three or more duplicate points (assay acceptance requires a standard curve with three or more duplicate points and an $R^2 > 0.95$); and
- No amplification in No Template Control (NTC) wells.

A small subset of sample extracts (IRP-AIR-10-24-11-ABC-018 to IRP-AIR-10-24-11-ABC-025 and sample IRP-AIR-10-24-11-ABC-27) was not qPCR-analyzed. This set of sample extracts was amplified on the ABI GeneAmp® 9700 PCR System (Life Technologies, Grand Island, NY, USA) and analyzed by gel electrophoresis, with direct visualization of the ethidium bromide-stained target amplicon. Positive and negative control reactions were prepared and analyzed

along with the sample extracts. Each sample was initially analyzed on a 2% agarose gel with 1X Tris base, acetic acid, and EDTA (TAE) running buffer (10 μL sample per well), and samples were electrophoresed on a 1.2% gel to compare pooled sample extracts (5 μL each) against pooled (No Template Controls) NTCs and the positive control reaction. Each gel contained an appropriate molecular weight marker, either Quick-Load 2-log ladder 2% gels (New England Biolabs, Ipswich, MA, USA) or 1Kb Plus Track It Ladder 1.2% gels (Life Technologies, Grand Island, NY, USA).

Sample extracts that did not pass IPC were subject to PCR using the Phire® Animal Tissue Direct PCR Kit (ThermoScientific, Waltham, MA, USA) (Table 11). Phire® PCR was conducted according to the manufacturer's instructions using pooled DNA extracts. Samples were pooled by combining 3 μL of each extract in groups of nine or ten. Reactions were created by combining 5 μL of each pooled sample extract with 25 μL 2X Phire® Animal Tissue PCR Buffer, 10.875 μL RNase-Free water (ThermoScientific, Waltham, MA, USA), 2.5 μL of each forward and reverse primer (10 μM), and 1 μL of Phire® Hot Start II DNA Polymerase. Reactions were processed with the following cycling parameters: initial denaturation (5 min, 98°C); 40 cycles of denaturation (98°C, 5 sec), annealing (65 °C, 5 sec), and extension (72°C, 20 sec); a final 1 min extension at 72°C. Each reaction was analyzed on 1.2% agarose gels; 25 μL of each PCR reaction was combined with 5 μL 6X Track It Loading Dye (Life Technologies, Grand Island, NY, USA) and run against the 1Kb Plus Track It Ladder.

3.5.2 Detection of PLGA Microspheres

During method development, a 96-well microtiter plate assay was developed for detection of PLGA microspheres in an aqueous extract. PLGA microspheres were diluted in 1X PBS to create a 10 mg/mL top concentration, which was then analyzed by dilution to extinction on two platforms: 1) SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA), and 2) Victor Fluorometer (PerkinElmer, Waltham, MA, USA) (0.1 and 1 sec exposure times). PLGA microspheres were analyzed in concentrations that ranged from 10 mg/mL to 1.19E-6 mg/mL (diluted 1:2 in 1X PBS). The working range of the SpectraMax M2 was determined to be 10 to 0.02 mg/mL whereas the working range of the Victor was 10 to 0.001 mg/mL. Due to the lower limit of detection obtained using the Victor fluorometer, that instrument was chosen for further assay development, and a standard curve was prepared and validated from 10 to 0.001 mg/mL.

Once the assay was established, verification of the proposed extraction method was initiated. Control sample matrices (sampling wipes, i.e., gauze and air filters) were spiked with 1 mg PLGA microspheres, and a mock extraction was performed according to project-specific work instructions (DWI-01-00; Appendix B). The PLGA microspheres were anticipated to be removed from the gauze and filter matrices and suspended in the extract, whereupon they would be recovered during the final filtration. However, the PLGA microspheres were discovered to be adsorbed to the gauze and filter matrices, and all attempts to remove them were unsuccessful. At the advice of the PLGA microsphere manufacturer, Phosphorex, Inc., 25 mL of a 2.5% solution of polyvinyl alcohol (PVA) was added to each spiked filter and gauze sample, followed by vortex agitation for 1 min. Room temperature incubation was continued up to 30 min with intermittent agitation by vortex. As no change was observed after 30 min, a water bath sonicator was used to agitate each sample for 5 min. Even after sonication in PVA, deposits of PLGA microspheres visible to the naked eye remained on both types of sample.

Table 11. Pooled Sample Extracts for Phire® Animal Tissue Direct PCR Kit

Pooled Sample	Sample Extracts Combined	Pooled Sample	Sample Extracts Combined
1	IRP-WIPE-10-19-11-ABC-B2 (QN) IRP-WIPE-10-21-11-ABC-0015 (QN) IRP-WIPE-10-21-11-ABC-0016 (QN) IRP-WIPE-10-21-11-ABC-0017 (QN) IRP-WIPE-10-21-11-ABC-0030 (QN) IRP-WIPE-10-21-11-ABC-0032 (QN) IRP-WIPE-10-21-11-ABC-0035 (QN) IRP-WIPE-10-21-11-ABC-0037 (QN) IRP-WIPE-10-21-11-ABC-0042 (QN)	4	IRP-WIPE-10-24-11-ABC-0072 (QN) IRP-WIPE-10-24-11-ABC-0073 (QN) IRP-WIPE-10-24-11-ABC-0074 (QN) IRP-WIPE-10-24-11-ABC-0075 (QN) IRP-WIPE-10-24-11-ABC-0076 (QN) IRP-WIPE-10-24-11-ABC-0077 (QN) IRP-WIPE-10-24-11-ABC-0078 (QN) IRP-WIPE-10-24-11-ABC-0080 (QN) IRP-WIPE-10-24-11-ABC-0081 (QN) IRP-WIPE-10-24-11-ABC-0096 (QN)
2	IRP-WIPE-10-21-11-ABC-0044 (QN) IRP-WIPE-10-21-11-ABC-0047 (QN) IRP-WIPE-10-24-11-ABC-0051 (QN) IRP-WIPE-10-24-11-ABC-0052 (QN) IRP-WIPE-10-24-11-ABC-0055 (QN) IRP-WIPE-10-24-11-ABC-0056 (QN) IRP-WIPE-10-24-11-ABC-0057 (QN) IRP-WIPE-10-24-11-ABC-0058 (QN) IRP-WIPE-10-24-11-ABC-0059 (QN) IRP-WIPE-10-24-11-ABC-0060 (QN)	5	IRP-WIPE-10-24-11-ABC-0083 (QN) IRP-WIPE-10-24-11-ABC-0084 (QN) IRP-WIPE-10-24-11-ABC-0086 (QN) IRP-WIPE-10-24-11-ABC-0087 (QN) IRP-WIPE-10-24-11-ABC-0088 (QN) IRP-WIPE-10-24-11-ABC-0089 (QN) IRP-WIPE-10-24-11-ABC-0090 (QN) IRP-WIPE-10-24-11-ABC-0092 (QN) IRP-WIPE-10-24-11-ABC-0093 (QN)
3	IRP-WIPE-10-24-11-ABC-0061 (QN) IRP-WIPE-10-24-11-ABC-0062 (QN) IRP-WIPE-10-24-11-ABC-0064 (QN) IRP-WIPE-10-24-11-ABC-0065 (QN) IRP-WIPE-10-24-11-ABC-0066 (QN) IRP-WIPE-10-24-11-ABC-0067 (QN) IRP-WIPE-10-24-11-ABC-0068 (QN) IRP-WIPE-10-24-11-ABC-0070 (QN) IRP-WIPE-10-24-11-ABC-0071 (QN) IRP-WIPE-10-20-11-ABC-001 (QN)		

Due to the apparent irreversible immobilization of PLGA microspheres onto the filter and gauze sample matrices, detection of PLGA microspheres was accomplished by direct microscopy using a Zeiss Axioscope epifluorescent microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a filter set with excitation at 495 and emission at 517 nm. Representative images were captured using a Zeiss color camera. More detail on these procedures can be found in Appendix B.

3.5.3 Enumeration of Putative Viable Bg in Archived Samples

Original air filter samples and archive gauze wipe samples were extracted according to the work instructions, DWI-01-02 (Appendix B). Samples were pre-wetted with 1X PBS extraction buffer (2 mL for filter samples, 5 mL for gauze samples) and mixed by vortexing for 30 seconds. An additional 10 mL of 1X PBS was added to each sample, and samples were incubated at room temperature (25±3 °C) for 30 min. Samples were mixed by vortexing for 0, 15, and 30 min. Following incubation, 200 µL of each sample was spread-plated onto BHIA and incubated

overnight at 30°C. Plates were observed for microbiological growth, and colonies were compared to an overnight positive control of *Bg* plated onto BHIA. Any samples containing putative *Bg* were replated onto fresh BHIA for enumeration. The putative *Bg* samples were diluted in 1X PBS and heat-shocked by incubation at 65°C for 30 min to kill any vegetative cells that might out-compete the spore-forming *Bg*. Positive and negative controls were processed along with the samples to ensure process efficacy. Negative controls were prepared by transferring clean filter and gauze matrices into sterile sample reservoirs; positive controls were prepared by transferring clean filters and gauze matrices into sterile sample reservoirs and spiking with an aliquot of *Bg*.

3.5.4 Identification of Background Microflora by Sequence Analysis

3.5.4.1 Selection of Unknown Isolates and Pooled Samples

Microorganisms recovered on BHIA from wipe and filter samples were selected for follow-on analysis using 16S rRNA sequencing. Thirty isolates that did not have morphology similar to *Bg* were selected and streaked for isolation on BHIA, followed by incubation for 16 – 48 hr at 36 ± 2 °C. Appendix A lists the isolate morphology and the sample from which the isolate originated. *Bg* (ATCC 9372) was included as a positive control.

Portions of the samples from each of the nucleic acid extract batches were combined to generate five pooled samples for metagenomic 16S rRNA analysis using the Ion Torrent™ Personal Genome Machine™ (PGM™) Sequencer (Life Technologies, Grand Island, NY, USA).

3.5.4.2 Extraction of DNA

Three different extraction techniques were used to prepare DNA for 16S rRNA amplification. Initially, each of the 30 isolates and five pooled samples was extracted following the DNeasy® Gram-positive bacteria protocol (Qiagen, Germantown, MD, USA); however, the DNeasy® extracts could not be used for PCR due to background 16S rRNA DNA that amplified in one of the enzymatic lysis buffer components. A thermolysis technique was therefore used to amplify the samples, but this technique too was unsuccessful at amplifying the 16S rRNA gene from the five pooled samples. Finally, a OneStep™ PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA, USA) was used on the pooled samples prior to PCR amplification. Only samples processed for sequencing were extracted using the OneStep™ PCR Inhibitor Removal Kit (Zymo). Inhibition testing was not attempted on these samples, as this kit was specifically designed for PCR and sequencing applications. Because all five pooled samples showed amplification after treatment on the OneStep™ column, no inhibition was presumed.

For extraction using DNeasy® Blood and Tissue Kit, enzymatic lysis buffer was prepared as follows: 2 mL of Tris-EDTA, 10X (Fisher Scientific, Pittsburgh, PA, USA), 120 µL of Triton X-100 (Fisher Scientific, Pittsburgh, PA, USA), and 2 mL of 100 mg/mL lysozyme, egg white (Fisher Scientific, Pittsburgh, PA, USA) was added to 5.88 mL of water. One to several colonies, depending on size, were selected for extraction. After addition of the colonies to a tube containing 180 µL of the above enzymatic lysis buffer, extractions were completed following the manufacturer's instructions for Gram-positive bacteria. To prepare pooled samples for extraction, 1 mL of each pooled sample was centrifuged at 5,000 x g for 10 minutes, and the pellet was suspended in 180 µL of enzymatic lysis buffer and extracted according to the manufacturer's instructions as stated above.

For extraction via thermolysis, DNA from the 30 isolates with distinct colony morphologies were extracted by adding one to several colonies, depending on size, to a tube containing 250 µL of 1X Tris-EDTA. The samples were autoclaved using a liquid cycle for 10 min at 121°C. Following autoclave treatment (121 °C for 10 min), the samples were cooled to room temperature and stored at -80°C until ready for use. The autoclave treatment step is a historical

method used at Battelle to remove DNA from intact cells, and it is not referred to as a published method. Five pooled samples were prepared by adding 10 µL of each pooled sample to a separate tube containing 250 µL of 1X Tris-EDTA and treating them in the same manner as the colony samples.

For extraction of PCR inhibitors using the OneStep™ column, fifty µL of each pooled sample was processed using the OneStep™ PCR Inhibitor Removal Kit following the manufacturer's instructions.

3.5.4.3 Amplification of 16S rRNA

The 30 isolated colonies and five pooled samples were subject to PCR using 8F (isolated colonies and pooled samples) or 27F (pooled samples) and 1492R 16S rRNA primers (Table 12).

Table 12. 16S rRNA Primer Sequences

Primer ID	Sequence
8F	5'-AGAGTTTGATCMTGGCTCAG-3'
27F	5,-AGAGTTTGATCCTGGCTCAG -3'
1492R	5'-GGYTACCTTGTTACGACTT-3'

A high-fidelity polymerase, Phusion™ (New England Biolabs, Ipswich, MA, USA) was used to amplify the 16S rRNA gene from each of the 30 isolated colonies. PCR of the 30 isolated colonies was carried out in a 50 µL total volume containing: 1 X Phusion™ High-Fidelity (HF) Buffer, 0.02 U/µL of Phusion™ DNA Polymerase, 0.5 µM of each primer, and 0.2 µM of each deoxyribonucleotide (dNTP) inoculated with 5 µL of thermolyzed colonies. Cycling conditions were carried out on an ABI 9700 thermocycler according to the following: an initial hold at 98°C for 30 sec; 35 cycles of denaturation (98 °C for 10 sec), annealing (55 °C for 30 sec), and extension (72 °C for 1 min); a final hold at 72 °C for 5 min. For samples amplified with primers 27F and 1492R, the annealing temperature was raised to 56 °C. PCR products were quantified by UV-absorbance using the NanoDrop™ 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA).

Initially, pooled samples were subject to PCR using primers 8F and 1492R, and then amplified using a polymerase with high resistance to many PCR inhibitors, Phire® (NEB) (New England Biolabs, Ipswich, MA, USA). The Phire® PCR was carried out in 50 µL total volume containing 1 X Phire® Animal Tissue PCR Buffer, 1 µL of Phire® Hot Start II DNA Polymerase, and 0.5 µM of each primer, inoculated with 5 µL of OneStep™ cleaned pooled sample. Cycling conditions were carried out on an ABI 9700 thermocycler with an initial hold at 98 °C for 5 min; 40 cycles of denaturation (98 °C for 5 sec), annealing (55 °C for 5 sec), and extension (72 °C for 40 sec); a final hold at 72 °C for 1 min. Following amplification of the 16S rRNA gene, the size of the amplified product was analyzed or visualized using 1.2 % Agarose E-Gel® (Life Technologies, Grand Island, NY, USA) and an E-Gel® 1 Kb Plus DNA ladder (Life Technologies, Grand Island, NY, USA).

A second amplification of the pooled samples was undertaken using the 27F and 1492R primers; no further amplification was required for these PCR products prior to sequencing.

3.5.4.4 Sequencing of 16S rRNA genes

For sequencing of 16S rRNA from isolated colonies using an ABI 3130 Genetic Analyzer (Life Technologies, Grand Island, NY, USA), the 16S rRNA PCR products generated from isolated colonies were purified using the GenElute™ PCR Clean-up Kit (Sigma-Aldrich, St. Louis, MO, USA), and the concentration of each PCR product was determined using the NanoDrop™ 2000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). Forward and reverse cycle

sequencing reactions were set up using the same 8F and 1492R PCR primers that yielded the original PCR product. Cycle sequencing was carried out using an ABI BigDye® Terminator v3.1 (Life Technologies, Grand Island, NY, USA) in 20 µL total volume containing: 4 µL of Ready Reaction Mix, 2 µL of BigDye Sequencing Buffer, 5 picomole primer, and 20 – 40 ng of 16S rRNA PCR product. Cycling conditions were carried out on an ABI 9700 thermocycler with an initial hold at 96 °C for 1 min; 25 cycles of denaturation (96 °C for 10 sec), annealing (50 °C for 5 sec), and extension (60 °C for 4 min). A positive control, pGEM®-3Zf(+), and NTC negative controls were included. Cycle sequencing reactions were purified using the ABI BigDye® XTerminator™ Purification Kit (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions.

Capillary electrophoresis was conducted on each purified cycle sequencing reaction using the ABI 3130 Genetic Analyzer with the run parameters shown in Table 13.

All raw sequencing files were imported into Sequencing Analysis Software v5.2 (Life Technologies, Grand Island, NY, USA) and analyzed using the KB™ basecaller (Life Technologies, Grand Island, NY, USA) to provide per-base quality value predictions. The KB basecaller assigns a quality value (QV) for each basecall. A QV value of 20 or greater was considered to be good quality, meaning that the probability that the base was miscalled is no greater than 1%.

Table 13 Applied Biosystems 3130 Genetic Analyzer Run Parameters

Specific Parameters	
Parameter	**Setting**
Template	BDx_StdSeq50_POP7
Oven Temperature	60 °C
Poly Fill Volume	5020 steps
Current Stability	5.0 Amps
Pre-Run Voltage	15.0 kVolts
Pre-Run Time	180 sec
Injection Voltage	1.6 kVolts
Injection Time	4 sec
Voltage Number of Steps	40 nk*
Voltage Step Interval	15 sec
Data Delay Time	480 sec
Run Voltage	8.5 kVolts
Run Time	6000 sec

* Number of voltage ramp steps to reach Run voltage

Six isolates were sequenced using the ABI 3130 Genetic Analyzer, and then the remaining sequencing was completed on the Ion Torrent PGM™ (Life Technologies, Grand Island, NY, USA). Sequencing on the traditional 3130 instrument requires an initial selection on solid media, which can significantly limit the number of bacteria examined in a population as the majority of environmental isolates are viable but nonculturable. Due to the massive parallel sequencing capabilities, acquisition of genome sequences from several organisms at once was possible without the bias of the primary culture. Additionally, sequence analysis on the Ion Torrent PGM™ is less expensive than traditional sequencing methods.

For sequencing of 16S rRNA amplified with primers 8F and 1492R using Life Technologies Ion Torrent Personal Genome Machine (PGM™), a total of fourteen 16S rRNA amplicons, including a positive and negative control, 1550 base pairs (bp) in length, were initially processed to create a sequencing library. Library preparation generated a pool of amplicons tagged with a specific molecular barcode that allowed multiplexing of samples for analysis on a single PGM™ semiconductor chip. The Ion DNA Barcoding 1-16 kit (Life Technologies, Grand Island, NY, USA) was used to prepare the library for the multiplexing experiment. Briefly, each of the 16S DNA amplicons separately underwent enzymatic shearing to fractionate the 1550 bp products. A purification step was performed using Agencourt® AMPure® magnetic particles (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions. Ion Barcode Adapters™ (Life Technologies, Grand Island, NY, USA) were ligated to the fragmented, purified DNA. An additional purification step was performed using the Agencourt® AMPure® magnetic particles to remove small molecular weight fragments. Following purification, additional PCR was performed to incorporate unique molecular barcodes onto the adapter-modified, fragmented DNA and further amplify each molecule. After PCR, a final purification step was performed using the Agencourt® AMPure® magnetic particles. Each molecule in the final bar-coded library preparation was approximately 180-210 bp in length, including amplicon sequence, adapter, and barcode. Individual reactions were measured using the NanoDrop® instrument to quantify DNA concentrations prior to pooling a portion of each reaction into a single mixed sample. The concentration of the mixed sample was measured again using the NanoDrop® to determine the library pool dilution required for sequencing.

To prepare the mixed barcoded sample for sequencing, clonal amplification was performed on the Ion OneTouch™ instrument (Life Technologies, Grand Island, NY, USA). Briefly, the mixed, barcoded library was combined with IonSphere Particles™ (ISPs) (Life Technologies, Grand Island, NY, USA) followed by clonal amplification in an oil emulsion PCR, which binds a single molecule to each particle and creates multiple copies of each particle-bound fragment. Immediately following clonal amplification, the particle-bound fragments were enriched using the Ion OneTouch ES™ instrument. This process removes unbound particles and unbound library fragments to enrich particle-bound fragments. At this point, a quality control check was performed, wherein a small amount of the enriched ISPs was quantitated using the Qubit® 2.0 Flourometer (Life Technologies, Grand Island, NY, USA) to determine the extent of enrichment. After enrichment, ISPs were loaded into an Ion 316™ chip (a single ISP per well) (Life Technologies, Grand Island, NY, USA), and sequencing was carried out according to manufacturer's instructions.

Because sequencing could not be performed using the amplicons generated using the 8F and 1492R primer pair, a substitution was made for the forward primer. The primer substitution resulted in amplification and high quality sequence data in all samples. For sequencing of 16S rRNA amplified with primers 27F and 1492R using Life Technologies Ion Torrent PGM™, qualitative and quantitative measurements of 16S amplicons were made using the Qubit dsDNA BR Assay Kit on the Qubit 2.0 fluorometer followed by analysis on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using DNA High Sensitivity chips. Following this procedure, 16S amplicon samples were fragmented using a Covaris S220 sonicator (Covaris, Inc., Woburn, MA, USA) to generate approximately 300 bp fragments. Fragmentation quality was assessed using an Agilent Bioanalyzer. Sequencing libraries were made using Ion Plus Fragment Library kit (Life Technologies, Grand Island, NY, USA) for 200 bp sequencing. Library quality was verified using the Agilent Bioanalyzer and the Qubit. Clonal amplification was performed on an Ion One Touch instrument using the Ion Xpress™ Template 200 Kit (Life Technologies, Grand Island, NY, USA). Enrichment for the ISPs was done on the Ion One Touch ES, and quantification of the percent templated ISPs was performed on the Qubit fluorometer. Sequencing was performed with 316 chips on an IonTorrent PGM sequencer using

the Ion Sequencing 200 kit. The IonTorrent Suite Server (1.5.1) performed base calling and output raw sequence data in FASTQ format.

3.5.4.5 Sequence Analysis of 16S rRNA genes

Sequence reads from the ABI 3130 with a length of greater than 200 bps and high quality base calls were subject to nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) [10], searching against the 16S microbial database. The BLASTn results with the highest maximum identity percentage were reported.

FASTQ files were loaded into CLCBio Genomics Workbench software V 4.9 (CLCBio, Cambridge, MA, USA). Trimming of sequence reads was performed to remove PCR primer sequences and low quality reads (0.05 quality threshold). For trimming, the quality values were used to establish a "clear range," bases from the ends of the sequence read were removed until fewer than four bases out of 20 had a QV of less than 20. A final filtering of reads was performed to select for reads of >175 bp. The National Institute of Health's National Center for Bioinformatics (NCBI) 16S rRNA (v6/15/2102) sequence database was loaded into CLCBio as a reference library. Two bioinformatics analyses were performed. First, read files were processed using the Battelle Galileo high performance compute cluster and the Basic Local Alignment Search Tool (BLAST®) (National Library of Medicine, Bethesda, MD, USA). Reads were searched against the NCBI 16S rRNA gene database (v6/15/2102) (NCBI, Bethesda, MD), which contained entries for 7,545 sequences. Search results were filtered for sequences with ≥97% identity. The output from this search resulted in a list of taxonomic identifications, associated organism names, and number of reads per taxID for each sample. Krona v. 2.1 [11] was used to create a comparative chart for viewing the relative abundance of organisms at the genus level for each sample. A final filtering of results was performed to include only taxa identified by numbers of hits greater than 0.1% (1:1000) of the total representation per sample. The second analysis, the Battelle QUEST™ tool, a recent research and development (R&D) using weighted probabilities based on genome coverage from reference aligned data, was used to measure the amount of individual reads mapping to each 16S rRNA sequence with the optimized parameters in CLCBio software and backend statistical analysis. The output was reported as most probable species present in the sample.

4. RESULTS

4.1 *Bg* Detection by Quantitative PCR

The qPCR analysis for *Bg* signatures was complicated by the co-extraction of inhibitory components from sample matrices. The extraction method (DWI-01-00; Appendix B) used for this project is a slight modification of a method that has been used extensively to extract and recover trace nucleic acids from environmental samples. Sample matrices successfully processed using this method (DWI-01-00; Appendix B) include water, soil, cellulose, food, and fabric compositions. Generally, any inhibitory components that are co-extracted with the DNA can be counteracted by dilution (1:5 or 1:10) of the sample extract in 1X TE. In this case, only approximately a third of the sample extracts could be analyzed Neat, 1:5, or 1:10. The remaining two-thirds of the sample extracts required further purification using a Qiagen QIAquick PCR purification kit. These samples were diluted and tested for inhibition at neat, 1:5, 1:10, and 1:20. Sample extracts that passed IPC were analyzed in duplicate for *Bg rtp* signatures at the highest concentration that passed IPC. All controls performed as expected (positive and negative), which demonstrate the validity of the methods used and that negative results are not an artifact of method performance. Table 14 shows the results of qPCR, including the analyzed dilution, the threshold cycle (Ct), and quantity in GC/5 µL.

Bg DNA was not detected in any of the sample extracts. Sample number IRP-WIPE-10-21-11-ABC-24 was first thought to be positive, but upon further investigation, the multicomponent plot showed that the fluorescent signal in those wells was extremely high, and true amplification did not occur. Samples that were inhibited at all tested dilutions (Neat, 1:5, 1:10, QN, Q5, Q10, and Q20) were subject to PCR using the Phire® Animal Tissue Direct PCR Kit after pooling DNA extracts into five composite samples comprised of nine or ten sample extracts (Table 9). Phire® PCR was unsuccessful at amplification under these conditions; no amplification was observed in any sample, including the positive control (1E4 GC/5 µL amplified standard). Because the positive control reaction did not amplify, the PCR conditions appeared to be sub-optimal, and it was not possible to determine from this analysis whether these inhibited samples contained *Bg* DNA. The results for a small subset of samples were inadvertently omitted from the Interim Report. These samples were also analyzed by Phire® Animal Tissue Direct PCR Kit, in duplicate reactions using the 7900HT, rather than in the sample pools as described above. These samples did not amplify, also likely due to inhibition of the Phire® polymerase (results are listed in Table 14). Note that the Sample identifications (IDs) listed in Table 14 include a notation for the type of sample, date of sample acquisition, and a sampling location (see Figures 4 and 5). See Appendix B for a complete description of how positive and negative controls were prepared (page 3 of Appendix B) and see page 4 of Appendix B for a description of how controls were processed for RT-PCR. See page 9 of Appendix B for preparation of positive controls for sequence analysis.

Table 14. Results of *Bg* qPCR Analyses

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
IRP-AIR-10-19-11-ABC-B1	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B2	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B3	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B4	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B5	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B6	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-19-11-ABC-B7	1:5	Undetermined	0	<LOQ Negative
		44.47	0.38	
IRP-AIR-10-19-11-ABC-B8	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-19-11-ABC-B1	Qiagen *** 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-19-11-ABC-B3	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-19-11-ABC-B4	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-19-11-ABC-B5	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-001	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-002	1:5	Undetermined	0	Negative
		Undetermined	0	
RP-AIR-10-20-11-ABC-003	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-004	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-005	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-006	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-007	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-20-11-ABC-008	1:5	Undetermined	0	Negative

Table 14. Results of Bg qPCR Analyses (Continued)

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
		Undetermined	0	
IRP-AIR-10-21-11-ABC-009	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-010	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-011	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-012	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-013	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-014	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-015	1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-016	Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-AIR-10-21-11-ABC-017	Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-IW-10-20-11_ABC-001	Neat	Undetermined	0	<LOQ Negative
		44.79	0.30	
IRP-WIPE-10-20-11-ABC-002	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-003	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-004	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-005	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-006	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-007	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-008	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-009	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-20-11-ABC-0010	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0011	Qiagen 1:5	Undetermined	0	Negative

Table 14. Results of Bg qPCR Analyses (Continued)

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0012	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0013	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0014	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0018	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-21-11-ABC-0019	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-020	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-021	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-022	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-023	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-21-11-ABC-0024	1:5	22.35	4.2E6	MC**** Negative
		19.84	1.45E7	
IRP-WIPE-10-20-11-ABC-025	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-026	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-027	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-20-11-ABC-0028	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-21-11-ABC-0029	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-21-11-ABC-0031	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0033	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0034	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0036	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0038	Qiagen 1:5	Undetermined	0	Negative

Table 14. Results of Bg qPCR Analyses (Continued)

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0039	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0040	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0041	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0043	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-21-11-ABC-0045	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0046	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0048	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-21-11-ABC-0049	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-050	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-053	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-054	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-063	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-24-11-ABC-069	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-079	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-24-11-ABC-082	Qiagen Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-085	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-091	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-094	Phire®	No Amplification	0	Undetermined
		No Amplification	0	
IRP-WIPE-10-24-11-ABC-095	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-097	Qiagen 1:20	Undetermined	0	Negative

Table 14. Results of Bg qPCR Analyses (Continued)

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-098	Qiagen 1:10	Undetermined	0	Negative
		Undetermined	0	
IRP-WIPE-10-24-11-ABC-099	Qiagen 1:20	Undetermined	0	Negative
		Undetermined	0	
IRP-FPG-10-24-11-ABC-001	Neat	Undetermined	0	Negative
		Undetermined	0	
IRP-FPC-10-24-11-ABC-001	1:5	Undetermined	0	Negative
		Undetermined	0	
Filter Blank 1	1:5	Undetermined	0	Negative
		Undetermined	0	
Filter Blank 2	1:5	Undetermined	0	Negative
		Undetermined	0	
Gauze Blank 1	1:5	Undetermined	0	Negative
		Undetermined	0	
Gauze Blank 2	Neat	Undetermined	0	Negative
		Undetermined	0	
Gauze Blank 3	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
Gauze Blank 4	1:5	Undetermined	0	Negative
		Undetermined	0	
Gauze Blank 5	Qiagen 1:5	Undetermined	0	Negative
		Undetermined	0	
Water Blank 1	Neat	Undetermined	0	Negative
		Undetermined	0	
Grease Blank 1	Neat	Undetermined	0	Negative
		Undetermined	0	
Filter PC 1	1:5	35.49	204.86	Positive
		35.09	272.53	
Filter PC 2	1:5	31.62	4821.90	Positive
		31.06	6351.25	
Gauze PC 1	1:5	32.65	1510.49	Positive
		32.64	1523.35	
Gauze PC 2	Neat	30.21	8087.46	Positive
		30.04	8778.85	
Gauze PC 3	Qiagen 1:5	32.21	3018.52	Positive
		32.65	2425.59	
Gauze PC 4	Qiagen 1:5	35.29	797.78	Positive
		34.91	963.46	
Gauze PC 5	Qiagen 1:5	38.43	171.44	Positive

Table 14. Results of Bg qPCR Analyses (Continued)

Sample ID	Dilution	Ct Value	GC/5 µL*	Result**
		35.78	628.65	
Water PC 1	1:5	31.37	3712.34	Positive
		31.69	2976.29	
Grease PC 1	Neat	32.93	1244.29	Positive
		32.98	1200.10	

* Gene copies per 5 µL of PCR reaction (after sample extraction, concentration by alcohol precipitation, re-suspension, etc.).
** Positive = > LOQ; LOQ was 92.1 GC/5 µL; samples with mean <1 GC/5 µL are considered Negative; samples with multicomponent trace showing no amplification are considered Multicomponent Negative.
*** These sample extracts were inhibited Neat, 1:5, 1:10, and 1:20. They were further purified by Qiagen kit and diluted to overcome inhibition.
**** MC = Multicomponent.

Sample extracts IRP-AIR-10-24-11-ABC-018 to IRP-AIR-10-24-11-ABC-025 and sample IRP-AIR-10-24-11-ABC-27 were amplified on the ABI 9700 instrument and analyzed by gel electrophoresis, with direct visualization of ethidium bromide-stained target amplicon (82 bp). Positive control reactions containing 1000 GC/5 µL standard control material and NTCs containing 1X TE were prepared and analyzed along with the sample extracts. No *Bg* DNA was detected in any of the samples or NTCs, but a faint band was observed in the 1000 GC/5 µL standard positive control well that could be consistent with the 82 bp amplicon (Figure 7). Note that in Figure 7 the blue arrow denotes presence of a faint amplicon at ~82 bp in the positive control well. Bands are visible in the NTC and pooled sample wells but are migrating slightly lower than the band in well 4 and may be primers. The amplification product in this figure was generated using the primers developed for the quantitative PCR reaction against *Bg rtp* gene (the Battelle assay), not the 8F and 1492R primers that were used for sequencing 16S rRNA. The amplicon for the *Bg* Battelle assay generates an 82 bp amplicon and was used in an attempt to detect amplification in samples processed using the Phire® Animal Tissue Direct PCR kit — these samples had shown inhibition after extraction, dilution, and Qiagen purification with subsequent dilution. The extensive inhibition was believed to be due to enzymatic and proteolytic activity of the animal tissues present in/on the sample.

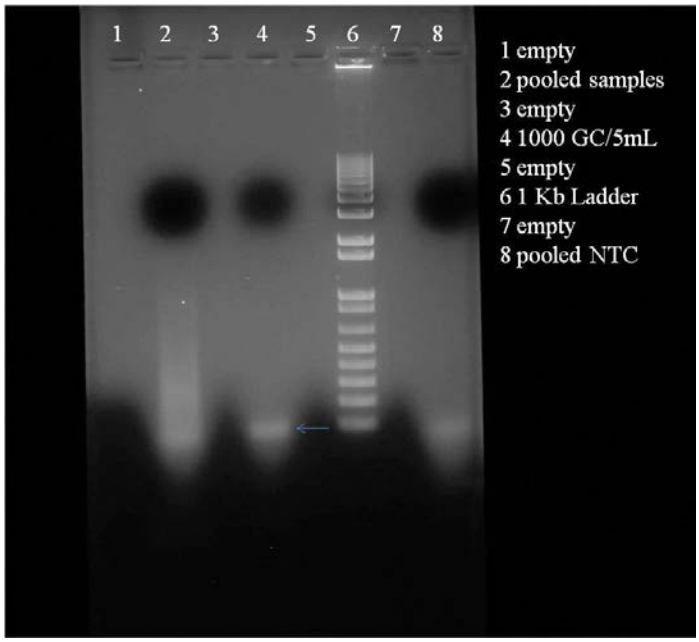

Figure 7. Gel Electrophoresis of AIR-10-21-11 Samples Analyzed by PCR on the ABI 9700 Thermocycler

4.2 Detection of PLGA Microspheres

PLGA microspheres were observed in all positive control samples but in low quantities (i.e., less than 20 microspheres per view). Sample autofluorescence prevented visualization of PLGA in most samples; only samples IRP-WIPE-10-24-11-ABC-0089 and IRP-WIPE-10-24-11-ABC-0099 contained fluorescent particles consistent with the PLGA microspheres. The gauze and filter matrices are autofluorescent, creating a diffuse green background under the epifluorescent conditions. Irregularly-shaped autofluorescent particulate matter in and on some sample matrices made it impossible to discern PLGA microspheres, if present. Table 15 lists each sample and the corresponding microscopic descriptions. Representative photos are also shown in Table 15. Several of the samples had begun to support mold growth at the time of microscopy, which also contributed to autofluorescence. Note that the Sample IDs listed in Table 15 include a notation for the type of sample, date of sample acquisition, and a sampling location (see Figures 4 and 5).

4.3 Enumeration of Putative Viable *Bg* in Archived Samples

After it was observed that the analyses of the original samples were not working well, the Test Team decided to process the archive samples in a new, different manner in hopes of getting better results. None of the air filter samples contained *Bg*, and no archival air samples were collected. Ten gauze wipe samples had putative *Bg* colonies (Table 16). Putative *Bg* was observed in six of these ten presumptive positives (i.e., putative) when plated for enumeration, although one sample displayed quantities <LOQ. The remaining four presumptive positive samples contained no putative *Bg* when plated for enumeration. These samples may contain *Bg* at levels approaching the LOD. Heat-shock was used to reduce the number of vegetative cells present in the sample extract dilutions. Extract dilutions plated prior to heat-shock (Table 16, Section A) had significantly fewer putative *Bg* colonies as compared to extract dilutions plated after heat-shock (Table 16, Section B). Heat-shock, therefore, aided in enumeration of the putative *Bg* colonies. The *Bg* positive control spikes were recovered at approximately the same concentration before and after heat-shock. *Bg* was recovered in the spiked air filter positive control, but not from any of the air filter test samples. Note that the Sample IDs listed in Table 16 include a notation for the type of sample, date of sample acquisition, and a sampling location (see Figures 4 and 5).

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-AIR-10-19-11-ABC-B1	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-19-11-ABC-B2	Diffuse green fluorescence, no PLGA microspheres	N/A
IRP-AIR-10-19-11-ABC-B3	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-19-11-ABC-B4	Diffuse green fluorescence, no PLGA microspheres (Note – photograph looks like a very dark green square; no features observed)	
IRP-AIR-10-19-11-ABC-B5	Dark field with diffuse green fluorescence; no PLGA microspheres	
IRP-AIR-10-19-11-ABC-B6	Diffuse green fluorescence, no PLGA microspheres	N/A
IRP-AIR-10-19-11-ABC-B7	Diffuse green fluorescence, no PLGA microspheres	N/A
IRP-AIR-10-19-11-ABC-B8	Diffuse green fluorescence, no PLGA microspheres	N/A
IRP-WIPE-10-19-11-ABC-B1	Two fluorescent particles observed, too large to be PLGA microspheres	N/A
IRP-WIPE-10-19-11-ABC-B2	One-two fluorescent particles observed, too large to be PLGA microspheres	N/A
IRP-WIPE-10-19-11-ABC-B3	No PLGA microspheres observed, background fluorescence	N/A
IRP-WIPE-10-19-11-ABC-B4	No PLGA microspheres observed, background fluorescence	N/A
IRP-WIPE-10-19-11-ABC-B5	No PLGA microspheres observed	N/A
IRP-AIR-10-20-11-ABC-001	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-002	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-003	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-004	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-005	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-006	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-007	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-20-11-ABC-008	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-21-11-ABC-009	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-21-11-ABC-010	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-21-11-ABC-011	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-21-11-ABC-012	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-AIR-10-21-11-ABC-013	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-AIR-10-21-11-ABC-014	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-21-11-ABC-015	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-21-11-ABC-016	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-21-11-ABC-017	Diffuse green background with no fluorescent particles	N/A
IRP-IW-10-20-11-ABC-001	Observed crystalline-like fluorescent shards and spherical fluorescent particles; none discernible as PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-001	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-002	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-003	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-004	Dark field with diffuse some fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-005	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-006	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-007	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-008	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-009	Light field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-20-11-ABC-0010	Light field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0011	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0012	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0013	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0014	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0015	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0016	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0017	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0018	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0019	Light field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0020	Light field with diffuse green fluorescence; no PLGA microspheres	N/A

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-WIPE-10-21-11-ABC-0021	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0022	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0023	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0024	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0025	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0026	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0027	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0028	Dark field with diffuse some fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0029	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0030	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0031	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0032	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0033	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0034	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0035	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0036	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0037	Very bright green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0038	Very bright green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0039	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0040	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-21-11-ABC-0041	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0042	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0043	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0044	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0045	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0046	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0047	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-21-11-ABC-0048	Diffuse green background with no fluorescent particles	N/A

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-WIPE-10-21-11-ABC-0049	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-050	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-051	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-052	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-053	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-054	Light field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-055	Light field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-056	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-057	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-058	Dark field with diffuse green fluorescence; no PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-059	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-060	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-061	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-062	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-063	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-064	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-065	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-066	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-067	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-068	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-069	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-070	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-071	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-072	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-073	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-074	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-075	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-WIPE-10-24-11-ABC-076	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-077	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-078	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-079	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-080	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-081	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-082	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-083	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-084	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-085	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-086	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-087	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-088	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-089	Dark background with diffuse green fluorescence, 1 fluorescent particle observed consistent with PLGA microsphere	N/A
IRP-WIPE-10-24-11-ABC-090	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-091	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-092	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-093	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-094	Dark background with green fluorescence, many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-095	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-096	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres	N/A
IRP-WIPE-10-24-11-ABC-097	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-098	Diffuse green background with no fluorescent particles	N/A
IRP-WIPE-10-24-11-ABC-099	Very diffuse green background, ~5 fluorescent particles observed consistent with PLGA microspheres	N/A
IRP-FPG-10-24-11-ABC-001	N/A	N/A
IRP-FPC-10-24-11-ABC-001	N/A	N/A
IRP-AIR-10-24-11-ABC-018	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-019	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-020	Diffuse green background with no fluorescent particles	N/A

Table 15. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations	Image
IRP-AIR-10-24-11-ABC-021	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-022	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-023	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-024	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-025	Diffuse green background with no fluorescent particles	N/A
IRP-AIR-10-24-11-ABC-027	Diffuse green background with no fluorescent particles	N/A
Filter Blank 1	Diffuse green background with no fluorescent particles	N/A
Filter Blank 2	Diffuse green background with no fluorescent particles	N/A
Gauze Blank 1	Diffuse green background with no fluorescent particles	N/A
Gauze Blank 2	Green fluorescent background, no PLGA microspheres or fluorescent particles	N/A
Gauze Blank 3	Very diffuse green background, no PLGA microspheres or fluorescent particles	N/A
Gauze Blank 4	Diffuse green background, no PLGA microspheres or fluorescent particles	N/A
Gauze Blank 5	Diffuse green background with no fluorescent particles	N/A
Water Blank 1	No fluorescent particles	N/A
Grease Blank 1	N/A	N/A
Filter PC 1	Many fluorescent PLGA microspheres observed in both membrane and batting layer. (Note – photograph looks like a very dark green square; no features observed)	
Filter PC 2	Very bright green background with ~20 PLGA microspheres visible on the membrane; no PLGA microspheres visible on the batting	N/A
Gauze PC 1	Some fluorescent PLGA microspheres observed; fewer than on Filter PC1	N/A
Gauze PC 2	Some fluorescent PLGA microspheres observed in background of green autofluorescence	
Gauze PC 3	Bright green background with one fluorescent particle suspected to be PLGA microsphere	N/A
Gauze PC 4	Some fluorescent PLGA microspheres observed (~eight) in diffuse green background	N/A
Gauze PC 5	Very bright green background, few PLGA microspheres observed	N/A
Water PC 1	Five fluorescent PLGA microspheres	N/A
Grease PC 1	N/A	N/A

N/A – Not available

4.4 Identification of Background Microbial Flora by Sequence Analysis

4.4.1 Extraction of DNA

Initially, each of the 30 isolates and five pooled samples were extracted following the DNeasy® Gram-positive bacteria protocol (Qiagen, Valencia, CA, USA). The DNeasy® extracts could not be used for PCR due to background 16S rRNA DNA in one of the enzymatic lysis buffer components. A thermolysis technique was used to reduce the number of reagents needed for extraction, thus reducing the likelihood of contamination. All samples were heat-shocked. This method worked well for five of the 30 isolates and resulted in a faint PCR product in eight other isolates. These eight faint PCR products were purified and amplified again using the same 8F and 1492R primers.

None of the pooled samples amplified when extracted using the thermolysis technique and Phusion™ polymerase. The pooled samples that were extracted by thermolysis, as well as an aliquot of each pooled sample that had not gone through an extraction method, were cleaned using an OneStep™ PCR Inhibitor Removal Kit (Zymo) and amplified using Phire® polymerase. Four of the five pooled samples that had been thermolyzed resulted in a PCR product after cleaning, and all five pooled samples that had no prior extraction procedure amplified after removing inhibitors.

4.4.2 Amplification of 16S rRNA

To isolate background microorganisms, 58 samples were plated onto BHIA and incubated overnight at 36 ± 2 °C with a positive control of *Bg*. Colonies resembling the *Bg* positive control were identified in 15 of the 58 samples (Table 17). Thirty colonies with variable morphologies not resembling *Bg* were selected for sequence analysis. The 16S rRNA gene was successfully amplified from only 13 of the 30 unknown isolates when using the 8F and 1492R primers. Of those 13 samples, only five resulted in a clean PCR product with a concentration of at least 13 ng/µL. All five of the isolates that resulted in a clean PCR product were able to be identified by sequencing using the ABI 3130.

The majority of the organisms that were isolated either could not be extracted using the thermolysis method or were not compatible with the 8F and 1492R primers. While 8F and 1492R primers are considered “universal primers”, they are probably not ideal for all bacterial species, and other “universal primers” that target the 16S rRNA gene could potentially be used to amplify a portion of the gene.

Although not confirmed by PCR, if the putative CFU that were identified based on colony morphologies (Table 17) are assumed to be *Bg*, there does appear to be a trend that the number of positive samples (not the enumerated CFU, because those results were not available for these samples) for the Post-Cleaning Phase samples (N=10) are significantly higher than the number of positive samples for the Post-Inoculation Phase samples (N=4). In addition, the locations within the plant where positive samples were found were much more widely dispersed throughout the plant in the case of the Post-Cleaning Phase samples. This observation suggests that the cleaning process using the steam and hot water has the potential to spread the contaminant around the plant, even if the cleaning process results in a reduction in the overall levels of contamination. The Sample IDs listed in Table 17 include a notation for the type of sample, date of sample acquisition, and a sampling location (see Figures 4 and 5).

4.4.3 Sequencing of 16S rRNA

4.4.3.1 Sequencing of 16S rRNA from Isolated Colonies using Applied Biosystems 3130 Genetic Analyzer

The BLASTn result with the highest maximum identity percentage and the top 25 BLAST® results, as well as the sequence information obtained, are described in Appendix B. Six of the 30 unknown isolates resulted in at least one high quality sequencing read. Isolates 4, 19, 22, and 29 are likely *Proteus* species, isolate 15 is likely a *Planomicrobium* species, and isolate 16 is likely a *Curtobacterium* species.

4.4.3.2 Sequencing of 16S rRNA Amplified with Primers 8F and 1492R using Ion Torrent PGM™

Initial sequencing on the PGM™ yielded poor results most likely due to failure of the library preparation. Poor quality 16S DNA amplicons and/or the possibility of carryover inhibitory components may have caused the library preparation to fail. The PGM™ functioned properly, and a successful run occurred. After examination of the run, summary evidence pointed to the likelihood that poor clonal amplification had occurred on the OneTouch™. The ISPs loaded correctly into the micron-sized wells, and all fluidics and semiconductor functions operated normally. However, template ISPs gave a reading of 8.23% on the Qubit® during the quality analysis check prior to sequencing. The percentage recommended to proceed with sequencing is >50%. Poor clonal amplification was potentially due to poor library construction in the presence of inhibitors that interfered with ligation of the molecular barcodes and adapters. This step is crucial for all other subsequent steps in the library preparation and sequencing. Sequencing reads generated on the PGM™ were of low quality; a quality filtration was performed on the reads using CLCGenomics Workbench software, but there were too few reads post-filtration to perform accurate BLAST® analysis or assembly. The reads remaining after filtration were not analogous to anything in the 16S database. No data were therefore obtained from the PGM™ analysis.

4.4.3.3 Sequencing of 16S rRNA Amplified with Primers 27F and 1492R using Ion Torrent PGM™

The 16S rRNA PCR strategy with 27F and 1492 primers was successful in producing amplicons from all five pooled samples. Pools 2 through 5 gave high quality sequence data resulting from IonTorrent sequencing. Pool 1 did not yield sufficiently high quality data, due either to the 16S amplicon quality (source sample influence) or sequencing library and sequencer quality (sequencing influence). Resequencing of pool 1 was not performed due to time and budget constraints.

Table 16. Enumeration of Putative *Bg* Colonies in Sample Extracts

A. Prior to Heat-Shock					
Sample Number	**Plated Dilution**	**Average Plate Count***			**Final Enumeration (CFU/mL)**
IRP-WIPE-10-20-11-D-001	1 x 10-1	0	0	0	0
IRP-WIPE-10-20-11-D-004	1 x 10-1	0	0	0	0
IRP-WIPE-10-20-11-D-006**	1 x 10-1	<30	36	114	7.50E2
IRP-WIPE-10-20-11-D-007	1 x 10-1	0	<30	<30	<LOQ***
IRP-WIPE-10-20-11-D-008**	1 x 10-1	0	<30	39	3.90E2
IRP-WIPE-10-21-11-D-0012**	1 x 10-1	0	0	0	0
IRP-WIPE-10-21-11-D-0013	1 x 10-1	0	0	0	0
IRP-WIPE-10-21-11-D-0024**	1 x 10-1	0	0	0	0
IRP-WIPE-10-21-11-D-0029**,****	1 x 10-5	0	0	0	0
IRP-WIPE-10-24-11-D-0059	1 x 10-1	0	0	0	0
Filter Positive Control	1 x 10-3	142	142	150	1.45E5
Gauze Positive Control	1 x 10-3	38	41	53	4.40E4
B. After Heat-Shock					
Sample Number	**Plated Dilution**	**Average Plate Count**			**Final Enumeration (CFU/mL)**
IRP-WIPE-10-20-11-D-001	1 x 10-1	0	0	0	0
IRP-WIPE-10-20-11-D-004	1 x 10-1	61	<30	47	1.08E3
IRP-WIPE-10-20-11-D-006**	1 x 10-1	159	175	148	1.61E3
IRP-WIPE-10-20-11-D-007	1 x 10-1	185	148	186	1.73E3
IRP-WIPE-10-20-11-D-008**	1 x 10-1	52	60	53	5.5E2
IRP-WIPE-10-21-11-D-012**	1 x 10-1	53	60	67	6.0E2
IRP-WIPE-10-21-11-D-013	1 x 10-1	0	0	0	0
IRP-WIPE-10-21-11-D-0024**	1 x 10-1	<30	0	0	<LOQ
IRP-WIPE-10-21-11-D-0029**,****	1 x 10-5	0	0	0	0
IRP-WIPE-10-24-11-D-0059	1 x 10-1	0	0	0	0
Filter Positive Control	1 x 10-3	119	110	113	1.14E5
Gauze Positive Control	1 x 10-3	29	32	36	3.23E4

* Individual plate counts <30 are not statistically significant; these counts are reported as <30 and are not included in the calculation for final enumeration.
** Originally reported in the January 18, 2012 Interim Report.
*** <LOQ = less than limit of quantitation.
**** Upon initial plating, this sample contained a lawn of putative *Bg*; upon inspection after subsequent dilution and plating, the microorganism did not appear to be *Bg*.

Table 17. Samples Containing Colony Morphologies Similar to *Bg*

Bg-Containing Samples	Sample Description
IRP-WIPE-10-20-11-ABC-002	Post inoculation; Tipping floor, 12 feet (ft) from pit
IRP-WIPE-10-20-11-ABC-003	Post inoculation; wall of auger
IRP-WIPE-10-20-11-ABC-006	Post inoculation; Tipping floor, 12 ft from pit wall
IRP-WIPE-10-20-11-ABC-008	Post inoculation; Tipping floor, 12 ft from pit wall
IRP-WIPE-10-21-11-ABC-0011	Post cleaning; Center-left of tipping floor near door
IRP-WIPE-10-21-11-ABC-0012	Post cleaning; Grinder wall; left side
IRP-WIPE-10-21-11-ABC-0014	Post cleaning; Between floor drains; in front of electrical panel
IRP-WIPE-10-21-11-ABC-0017	Post cleaning; End of railroad tracks
IRP-WIPE-10-21-11-ABC-0018	Post cleaning; Three ft south of western tallow tank
IRP-WIPE-10-21-11-ABC-0019	Post cleaning; Office door; cooker room
IRP-WIPE-10-21-11-ABC-0024	Post cleaning; two ft from small crax grinder control panel
IRP-WIPE-10-21-11-ABC-0026	Post cleaning; Center of doorway near M3 tallow tank
IRP-WIPE-10-21-11-ABC-0027	Post cleaning; walkway, four ft from stairs near SS5 tank
IRP-WIPE-10-21-11-ABC-0029	Post cleaning; six in from drain near maintenance roll-up door

Table 18 shows the dominant genera of bacteria identified by BLASTn search and the most probable species identified by the Battelle QUEST™ method. Figures 8-11 present hierarchically organized relative abundance data resulting from Ion Torrent PGM™ sequence analysis using the KRONA tool. KRONA is an open source software built with HTML5 (web-browser format) that may ingest BLAST® data and prepare visual results of the relative abundances of the total top BLAST® hits. The KRONA maps in Figures 8-11 show resolution at the genus level (outer ring of the circle) organized to lower sub-classifications (inner radii of the circle). Percentages of BLAST® reads matching each group of bacteria is included in the figure to assist in interpretation. In general, all pools had *Pseudomonas* as the most prevalent genus, ranging from 31-87% of the total genetic sequences identified (Table 18). Pool five was the least diverse sample with *Pseudomonas* and *Shewanella* species comprising 95% of the sample. Other genera of bacteria discovered in the pools included *Stenotrophomonas*, *Xanthomonas*, *Comomonas*, *Herbaspirilium*, *Lactobacillus*, *Acinetobacter*, and *Yersinia*. The genus *Bacillus* was not observed in pools 2, 4 and 5 and was at a level near to the limit of detection for the methods used in pooled sample 3 (0.04% of the genetic material identified). Further, most of the species identified from pools 2-5 belonged to the phylum *Proteobacteria*, with low observance (<5%) of the phyla *Firmicutes*, *Bacteroidetes* and *Actinomycetales* (Figures 8-11). In general, the pools had similar profiles of bacteria identified by 16S sequencing, varying mostly by abundance of genera between pools.

Amplification of 16S rRNA genes was accomplished in only 13 of 30 attempted reactions from the isolated colonies, and sequence analysis of the 16S rRNA genes was achieved for only six of these 13 amplicons. The remaining seven amplicons were likely of poor quality and not suitable for sequence analysis. Amplification of 16S rRNA genes is performed using 'universal' primers that are generated to conserved regions in the 16S genes. However, there are several sets of primers that can be used, and PCR conditions may favor certain amplicons over others. If a different primer set is chosen, additional isolates may be identified.

Sequence analysis of the pooled sample extracts was improved using primers 27F and 1492R (as compared to primers 8F and 1492R). *Pseudomonas* was the primary genus present in sample pools 2 through 5. Sequence analysis could not be performed on pool 1; the 16S amplicons were of insufficient quality.

Table 18. Results of 16S rRNA Sequencing Based on BLAST® and QUEST™ Analysis

Sample	Dominant Genera by BLAST®	Dominant Organisms by QUEST™ (top 15 most probable species)
Pool 1	ND*	ND
Pool 2	*Pseudomonas* (48%) *Stenotrophomonas* (18%) *Xanthomonas* (5%) *Yersinia* (4%) *Comamonas* (4%)	Stenotrophomonas_rhizophila_strain_e-p10 Pseudomonas_fragi_strain_ATCC_4973 Acidaminococcus_intestini_strain_ADV_255.99 Stenotrophomonas_maltophilia_strain_IAM_12423 Acidaminococcus_fermentans_strain_VR4 Comamonas_kerstersii_strain_LMG_3475 Simplicispira_metamorpha_strain_DSM_1837 Comamonas_aquatica_strain_:_LMG_2370 Pseudomonas_psychrophila_strain_E-3 Microvirgula_aerodenitrificans_strain_Sgly2 Pseudomonas_lundensis_strain_ATCC_49968 Stenotrophomonas_koreensis_strain_TR6-01 Dysgonomonas_capnocytophagoides_strain_LMG Pseudomonas_agarici_strain_71A Brevundimonas_terrae_strain_KSL-145
Pool 3	*Pseudomonas* (31%) *Shewanella* (18%) *Acinetobacter* (7%) *Herbaspirillium* (6%) *Stenotrophomonas* (4%) *Lactobacillus* (3%)	Shewanella_baltica_strain_63 Stenotrophomonas_rhizophila_strain_e-p10 Pseudomonas_fragi_strain_ATCC_4973 Herbaspirillum_autotrophicum_strain_IAM_14942 Shewanella_morhuae_strain_U1417 Morganella_psychrotolerans_strain_U2/3 Herbaspirillum_rhizosphaerae_strain_UMS-37 Paucimonas_lemoignei_strain_LMG_2207 Acinetobacter_ursingii_strain_LUH Arcobacter_nitrofigilis_strain_CI Dysgonomonas_capnocytophagoides_strain_LMG Lactobacillus_curvatus_strain_:DSM_20019 Shewanella_putrefaciens_strain_LMG_26268 Myroides_odoratimimus_strain_:_CCUG_39352 Acinetobacter_haemolyticus_strain_DSM_6962

Table 18. Results of 16S rRNA Sequencing Based on BLAST® and QUEST™ Analysis

Sample	Dominant Genera by BLAST®	Dominant Organisms by QUEST™ (top 15 most probable species)
Pool 4	*Pseudomonas* (34%) *Stenotrophomonas* (42%) *Xanthomonas* (10%) *Pseudoxanthomonas* (3%)	Stenotrophomonas_rhizophila_strain_e-p10 Pseudomonas_fragi_strain_ATCC_4973 Stenotrophomonas_koreensis_strain_TR6-01 Stenotrophomonas_maltophilia_strain_IAM_12423 Pseudomonas_hibiscicola_strain_ATCC_19867 Pseudomonas_psychrophila_strain_E-3 Stenotrophomonas_nitritireducens_strain_L2 Pseudomonas_geniculata_strain_ATCC_19374 Pseudomonas_mucidolens_strain_IAM12406 Pseudoxanthomonas_spadix_strain_IMMIB_AFH-5 Mycoplana_bullata_strain_IAM_13153 Stenotrophomonas_terrae_strain_:_R-32768 Pseudomonas_extremorientalis_strain_KMM_3447 Pseudomonas_abietaniphila_strain_:ATCC_700689 Pseudomonas_moraviensis_strain_CCM_7280
Pool 5	*Pseudomonas* (87%) *Shewanella* (8%)	Pseudomonas_fragi_strain_ATCC_4973 Pseudomonas_agarici_strain_71A Shewanella_putrefaciens_strain_LMG_26268 Pseudomonas_psychrophila_strain_E-3 Shewanella_baltica_strain_63 Pseudomonas_lundensis_strain_ATCC_49968 Pseudomonas_veronii_strain_CIP_104663 Pseudomonas_libanensis_strain_CIP_105460 Stenotrophomonas_rhizophila_strain_e-p10_ Pseudomonas_palleroniana_strain_CFBP_4389 Shewanella_hafniensis_strain_P010 Shewanella_oneidensis_strain_MR-1 Pseudomonas_mucidolens_strain_IAM12406 Pseudomonas_caricapapayae_strain_Robbs_ENA-378 Pseudomonas_taetrolens_strain_I11

*ND = no data

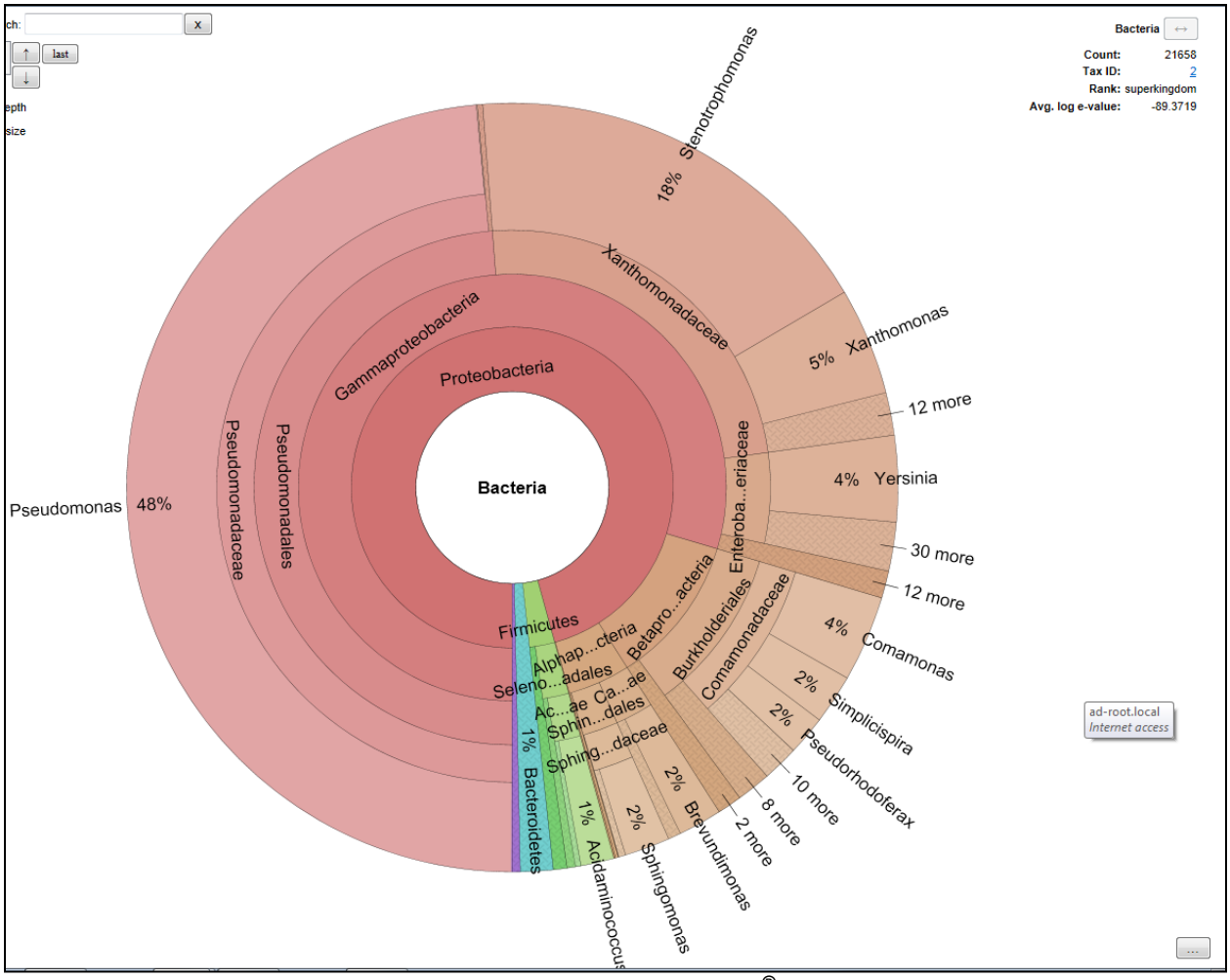

Figure 8. KRONA Visualization of BLAST® Results for Pool 2

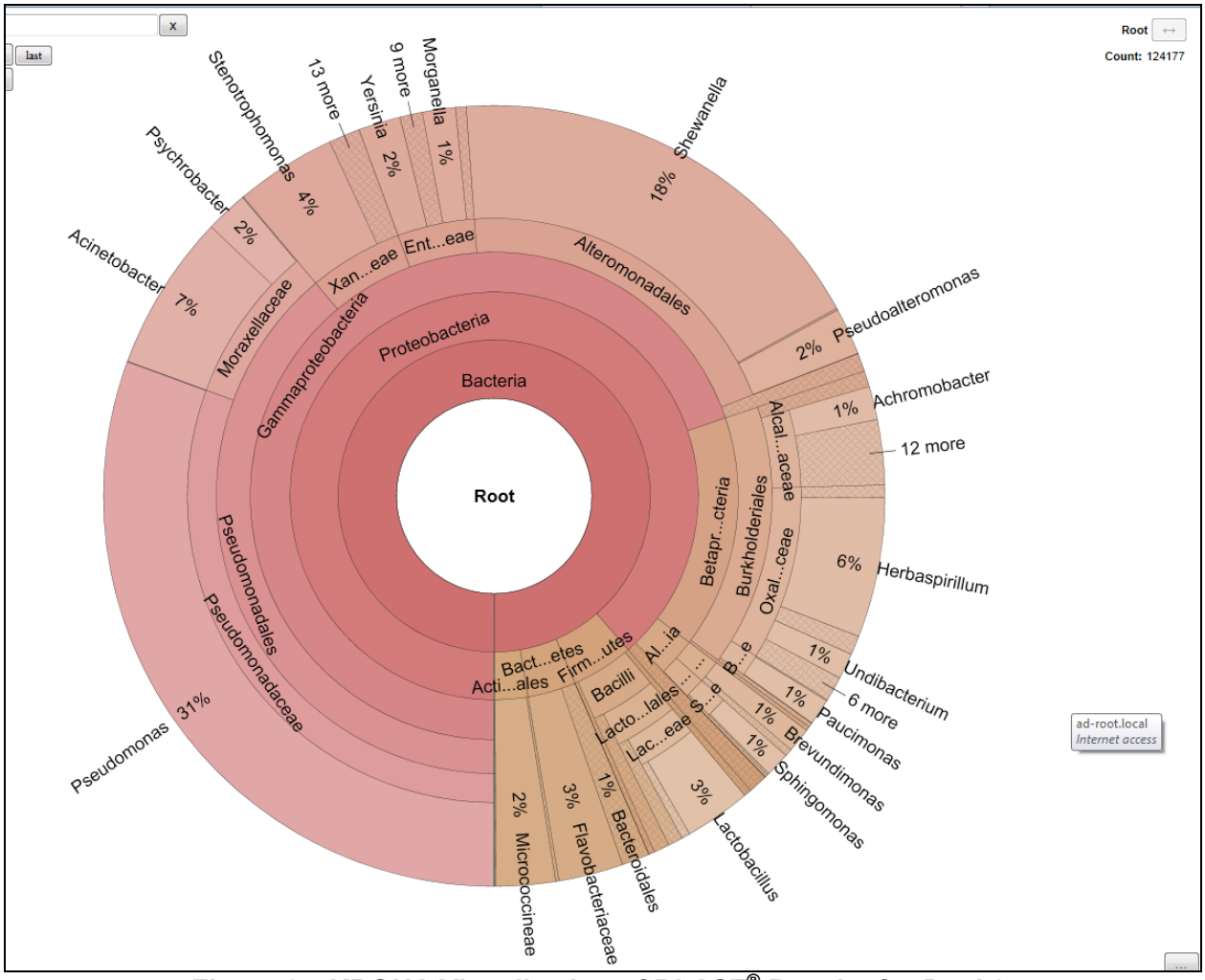

Figure 9. KRONA Visualization of BLAST® Results for Pool 3

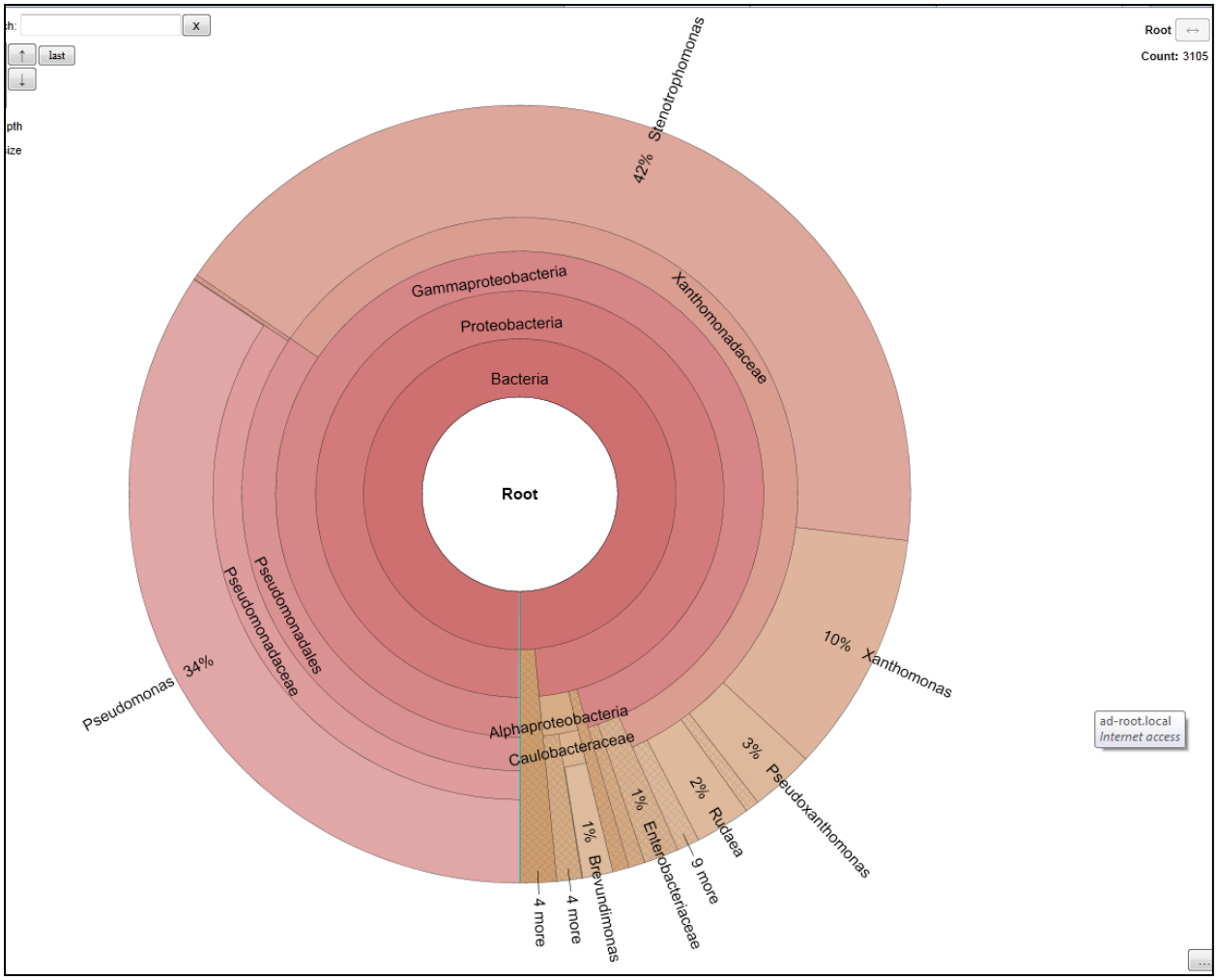

Figure 10. KRONA Visualization of BLAST® Results for Pool 4

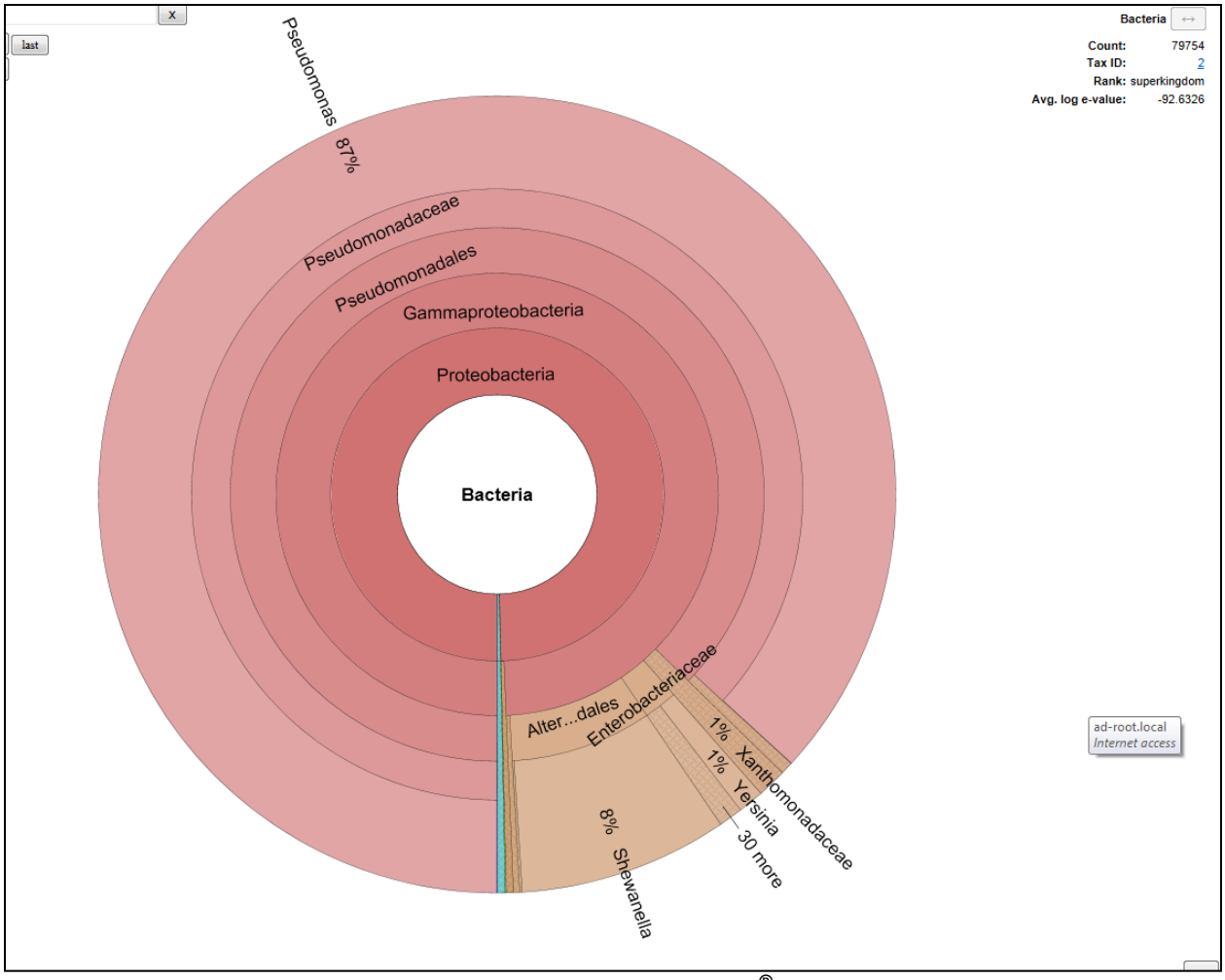

Figure 11. KRONA Visualization of BLAST® Results for Pool 5

4.5 Summary of Results

Quality control samples and spiked samples were analyzed for *Bg* DNA. All the positive controls came back positive. All the negative controls were negative (there was only one and it was the distilled water). *Bg* DNA was not detected in any of the sample extracts collected from the rendering plant from either the surface wipe samples or the air samples. However, viable bacteria very similar to *Bg* positive control colony morphology were recovered from 15 of 58 samples that the original sample extracts were created from (air samples B1-B8, air samples 001-025 and -027, wipe samples 001-024), and from ten of the archived test samples (five contained putative *Bg* in quantities greater than LOQ). Samples with one or more individual plate counts <30 were reported as <LOQ (individual plate counts <30 are not considered statistically significant).

A significant amount of additional effort was given to extracting *Bg* DNA from the samples, including sacrificing all of the archived samples in an attempt to use alternate means to amplify the *Bg* DNA and achieve detection. This additional effort was unsuccessful.

The sample results indicated that *Bg* DNA could be extracted from, and detected in, spiked positive controls of pristine gauze and air filter matrices prepared from the same lots of gauze wipe and air filters as the samples. *Bg* may have been present in low quantities and/or below the LOD by qPCR; however, nondetection by qPCR was more likely due to inhibitors such as

grease, bone, hair, etc., that carried over during the extraction process because *Bg* was recoverable on BHIA.

Detection of *Bg* DNA via PCR techniques might possibly be improved by dramatically increasing the concentration of *Bg* in the inoculum. However, the concentration is likely to need to be increased by one or more orders of magnitude, which may rapidly become cost-prohibitive for field testing of full-scale facilities. Because the heat shocking techniques did appear to improve detectability of *Bg* via conventional nonmolecular microbiological techniques, the use of heat-shock should be standard procedure for enumeration of rendering plant samples when using *Bg* as a surrogate.

The results of the analyses indicated that PLGA microspheres may not be a suitable synthetic surrogate. The microspheres appear to become immobilized in the sampling matrices, and the particles autofluoresce at a wavelength similar to hair and bone fragments, thus making the PLGA spheres difficult to distinguish from background. Also, the extraction processes were ineffective for removing PLGA microspheres for quantitation by fluorometer, and autofluorescence from the sample matrices complicated detection of PLGA microspheres via direct microscopic observation. Use of different-colored PLGA spheres, which may not autofluoresce at the same wavelengths as the materials in the sampling matrices, may be possible, although use of different-colored PLGA spheres would require additional methods development work.

Although it is difficult to perform a purely quantitative complete assessment of these results because the PCR was unsuccessful at reliably recovering *Bg* DNA from the samples, conventional nonmolecular microbiological methods appeared to succeed. If the putatively identified *Bg* CFU in Table 16, Section B are examined as a whole, the enumerated CFU from the samples taken on October 20, 2011 (the Post-Inoculation Phase sampling) appear to be approximately an order of magnitude or more higher than the enumerated CFU from the samples taken on October 21, 2011 (the Post-Cleaning Phase sampling). This difference suggests that routine plant cleaning procedures may potentially result in a 1-log reduction in pathogen loading within the potentially contaminated areas of the plant. Sufficient data to perform a statistical analysis on these results were lacking. This reduction in pathogen loading is not inconsistent with results from previous systematic studies examining the effectiveness of different steps of a multi-step cleaning/disinfection process that showed a 1-4 log reduction from individual cleaning/disinfection steps [12], although this reduction should be treated only as semi-quantitative in nature because the samples that showed putative *Bg* colonies were not always found at collocated sampling locations, and recovery of *Bg* spores from the sample media was poor. The plant cleaning procedures used in this study utilized hot water and steam, which would have been expected to remove contamination from surfaces and transfer any removed contamination into the rinse water going into the drains, as opposed to actually killing any pathogens that may have existed in the rinsate.

Although not confirmed by PCR, if the putative CFU that were identified based on colony morphologies (Table 17) are assumed to be *Bg*, there does appear to be a trend that the number of positive samples (not the enumerated CFU because those results were not available for these samples) for the Post-Cleaning Phase samples (N=10) are higher than the number of positive samples for the Post-Inoculation Phase samples (N=4). In addition, the locations within the plant where positive samples were found were much more diverse in the case of the Post-Cleaning Phase samples. Figure 12 illustrates these observations. Although the most heavily contaminated area (the Pit) did not show putative *Bg* colonies after cleaning, the cooker area and other areas that are not typically directly exposed to the raw material showed putative *Bg* colonies.

These observations suggest that the cleaning process using the steam and hot water has the potential to spread the contaminant throughout the plant, even if the cleaning process results in a reduction in the overall levels of contamination. It is not entirely clear whether this spread of contamination is the result of plant personnel tracking the surrogate to various locations within the plant or due to aerosol transport. High pressure spraying operations have indeed been shown to result in aerosol transport of spores [13]; however, in this study, no air samples exhibited any *Bg,* either through PCR analysis or examination of colony morphology.

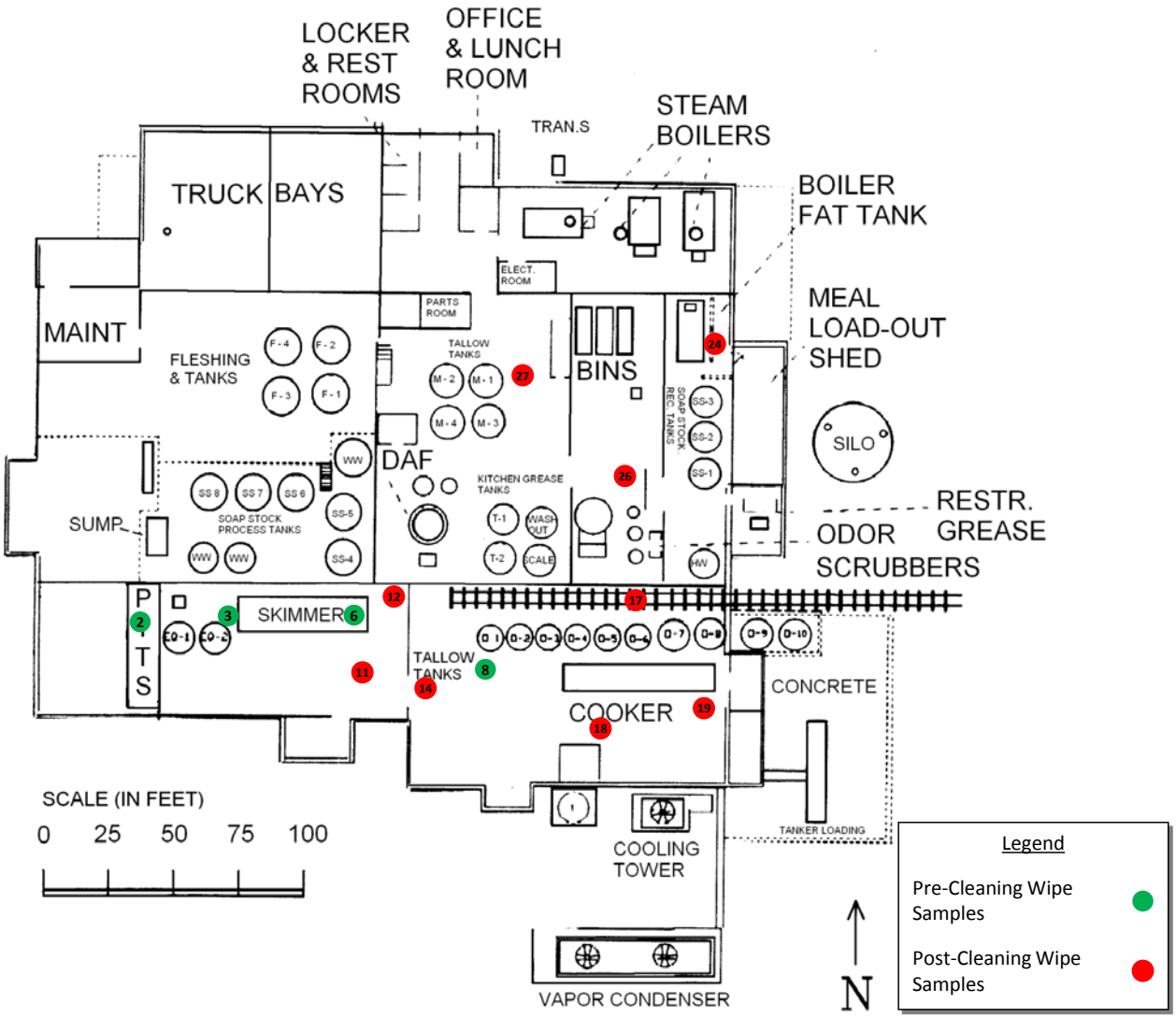

Figure 12. Locations of Putative *Bg* Colonies Before and After Cleaning

5. QUALITY ASSURANCE/QUALITY CONTROL

This effort attempted to achieve three objectives to reach the overall stated goal of evaluating cleanup of a rendering plant following its use for disposal following an FAD outbreak:

- To generate data on fugitive emissions of a biological surrogate during the rendering process;
- To determine the effectiveness of plant cleaning procedures for reducing the surrogate levels on the inside surfaces of the rendering facility; and
- To provide information that could be used to develop standard procedures for appropriately clearing a rendering facility that has been used for "disposal rendering" after an FAD outbreak so that the rendering facility can be returned to normal production.

An external review was not performed. However, an internal independent QC review was performed by the laboratory prior to delivery of results to the EPA Test Team.

5.1 Experimental Approach

Environmental characterization, decontamination, and clearance are critical components of a comprehensive public health recovery strategy in the aftermath of an FAD outbreak or a biological agent terrorist incident. This study looked to investigate the unique environment of a rendering plant and the rendering process that could play a critical role in the nation's response to an FAD event by assisting in the control of diseases and providing a mechanism to recycle usable animal carcasses into safe and usable products.

As one step toward addressing the clearance goals for returning a rendering plant back to normal operation, the EPA, the U.S. Department of Agriculture, and private industry worked together to evaluate fugitive emissions of a biological surrogate released from a rendering process. The evaluation process included characterizing the native bacterial flora of a rendering plant, determining a suitable biological surrogate, pre-release sampling, decontamination of the rendering facility, and post-decontamination sampling.

5.2 Sampling Approach

Sampling activities were conducted according to the Quality Assurance Project Plan (QAPP) that was approved prior to testing (available upon request). In several instances, sample numbers and locations identified in the QAPP were adjusted to meet changes in the original schedule or identifying valuable additional samples. Air samples (34) and surface and equipment samples (90) were collected from 124 sample locations. An additional 26 (twenty percent ratio) QA/QC samples were collected, including media blanks, duplicates, positive controls and field blanks. The original planned approach (See Table 6) outlined a total of 120 samples from the air, surfaces, and equipment throughout the Darling rendering plant. The additional samples were collected from air, grease (1) and crax (1). The grease and crax samples were opportunistic samples that were collected from the final rendering products. The additional air samples were collected to ensure that the inoculation process did not contaminate the rendering plant. Table 19 lists a summary of the sampling and analytical procedures.

5.2.1 Wipe Sampling

A slight deviation was made from the QAPP in the number of wipes that were collected and sent to the laboratory for analysis. The original plan called for three separate wipes from each of the surface sampling locations. However, laboratory personnel reduced the number of wipes because they could conduct Q-PCR, enumeration and PLGA identification from one wipe. The reduction saved on costs, reduced analysis time, and allowed the sampling team to designate a

second wipe for archival purposes.

Table 19. Summary of the Sampling and Analytical Procedures

Matrix	Measurement	Sampling/ Measurement Method	Sample Container/ Quantity of Sample	Preservation/ Storage	Holding Time(s)
Wipe	Culture and counts of CFU	Colony Counts per 100 cm^2	25 mL Conical Tube	Less than 25 °C	3 days
Wipe	PCR	Cycle time	25 mL Conical Tube	Less than 25 °C	3 days
Wipe	Immunoassay or spectrography for PLGA	Concentration	25 mL Conical Tube	Less than 25 °C	3 days
MCE Filter	Culture and counts of CFU	Colony Counts per 100 cm^2	37 mm filter in plastic cassette	Less than 25 °C	3 days
MCE Filter	PCR	Cycle time	37 millimeter (mm) filter in plastic cassette	Less than 25 °C	3 days
MCE Filter	Immunoassay or spectrography of PLGA	Concentration	25 mL Conical Tube	Less than 25 °C	3 days

5.2.2 Air Sampling

Air sampling was conducted in accordance with the QAPP. A slight deviation was made in the number of air samples collected. Additional air samples were collected to ensure that the inoculation process did not contaminate the rendering plant. Also, positive controls were collected that were not previously listed in the QAPP.

5.3 Timeline of Events for Study

The timeline of events was modified slightly from the QAPP due to budgetary concerns and plant operations. Oversight was not conducted by the Test Team during pre-cleaning during weekend three to reduce costs. Test personnel did tour the plant following the cleaning and prior to sampling and collected photographs to confirm that background cleaning had occurred properly. Also, a full grinder study that was planned for Monday, October 24th, could not be conducted due to budgetary concerns and plant maintenance operations.

5.3.1 Background Sampling

Background sampling was conducted in accordance with the QAPP. Following pre-cleaning, a total of 11 samples (four surface samples and seven air samples) were collected throughout the plant as background samples prior to the inoculation of incoming loads with the PLGA and *Bg*.

5.3.2 Carcasses Inoculated with PLGA and Bg

PLGA and *Bg* were sprayed throughout each truckload of carcasses intended for processing in the rendering plant for an eight-hr shift. The PLGA and *Bg* spores were sprayed on the carcasses using a Roundup backpack-style hand sprayer. The carcasses were inoculated in

accordance with the QAPP, although a slight variation in the mixture was utilized after the manufacturer of the *Bg* spores recommend using 1E9 *Bg* concentration and a surfactant to prevent clumping. According to the manufacturer, 1E11 *Bg* concentration is prone to clumping and using the lower (1E9) concentration with a surfactant is the optimum concentration to be sprayed.

5.3.3 Process Sampling

Process sampling was conducted in accordance with the QAPP, though one of the sample locations for the grinder was moved due to inaccessibility. During the processing of inoculated material in the eight-hr shift, sampling personnel collected two grab samples every two hours during the eight hr of processing (eight grab samples total). Originally, one of the samples was to be collected from the bottom of the tipping floor pit, and a second sample was to be collected from the incline screw leading from the pit. However, the pit was inaccessible from the top, so the sample was moved to the outer edge of the tipping floor pit. This location was chosen as an acceptable alternative because processing material was routinely pushed up against it with a front end loader.

5.3.4 Inoculation Phase and Process Sampling

Inoculation Phase and process sampling were conducted in accordance with the QAPP. Samples were collected from 28 sampling locations (six air sampling locations and 22 surface sampling locations).

5.3.5 Post-Inoculation and Process Sampling

Post inoculation and process sampling were conducted in accordance with the QAPP, although one of the sample locations for the grinder was moved due to inaccessibility. After the eight-hr shift when all of the contaminated carcasses were processed, the plant processed uninoculated carcasses for eight hr. During the Process Sampling, the sample location from the bottom of the tipping floor pit was moved due to the inaccessibility of the outer edge of the tipping floor pit. This same location was chosen for the Post-Process Sampling.

5.3.6 Plant Cleaning After Inoculation and Process Sampling

Following the sampling, plant personnel cleaned the facility according to the QAPP. Under the oversight of EPA, plant personnel utilized existing plant methods to clean the plant. Particular attention was paid to the grinder area, tipping floor, pits, the processing area, and building floors.

5.3.7 Post-Cleaning Sampling

After the cleaning was performed by plant personnel, test personnel performed sampling in accordance with the QAPP. Eighty wipe samples were collected from forty sample locations. One wipe sample was collected for analysis and a second wipe sample was collected for archiving. Sampling personnel collected eight air samples from areas where contamination and dust would most likely occur. The previously mentioned opportunistic samples of grease and crax were collected in this sampling phase to determine if the final rendering products showed any signs of contamination. In addition, numerous QA/QC wipe and air samples were collected in accordance with the QAPP.

5.3.8 Grinder Study Sampling

The full grinder study described in the QAPP was not conducted after final cleaning of the plant due to budgetary concerns and plant maintenance. The grinder study was designed to determine if new rendering material could possibly re-contaminate the plant from inoculum potentially left inside the grinder. However, limited samples were collected from the grinder during post-decontamination sampling. Data are therefore available for the grinder. In addition, samples were collected in each stage of the study from the grinder.

5.4 Analytical Procedures

As discussed in Section 4 (Results) of this report, the analytical laboratories had difficulties with the rendering matrix. Some modifications were therefore made to the analytical procedures (Table 17). As previously stated, sampling personnel collected two wipe samples from each of the surface sampling locations. One wipe (designated as ABC) was collected for community characterization by qPCR, enumeration, and PLGA identification. The second sample was collected and stored for archival purposes (designated as D). Sampling personnel collected samples of air PLGA and *Bg* using MCE filters and a sample pump.

All samples, including a *Bg* spike, were submitted to Battelle for bacterial identification (PCR), enumeration (counts), and analysis for PLGA microspheres (Victor fluorescence assay or fluorescent microscopy). Bacterial identifications were conducted on all samples using qPCR to determine the quantity of *Bg* and further processing, sequencing, and analysis utilizing 16S (and/or 23S) rRNA sequencing to identify other community microorganisms. Each wipe or filter sample was extracted using a standard procedure developed for recovery of viable microorganisms and nucleic acids. See Appendix B for more discussion on the procedures utilized during analysis.

A method for accurately enumerating PLGA fluorescent microspheres was developed in a 96-well format for the Victor fluorescence plate reader. A standard curve was prepared and evaluated in triplicate to determine the LOD and LOQ for the assay. Additionally, gauze and filter samples were spiked with a known quantity of PLGA microspheres and extracted according to the proposed method (filtration and recovery as described above) and enumerated against the standard curve to verify the procedure.

Viable bacteria were compared to a control culture of *Bg* on BHI agar, and colonies that did not have morphology similar to *Bg* were selected for follow-on analysis to amplify and sequence the 16S-23S rRNA gene. Up to thirty isolates with unknown colony morphologies were selected for follow-on analysis. Briefly, a single colony was placed into a PCR reaction tube along with 50 µL of Promega nuclease-free water (VWR, PAP1195, West Chester, PA, USA) with a sterile inoculating loop and autoclaved for 15 min. at 121°C. Each colony selected was analyzed by sequencing the 16S rRNA gene. The 16S rRNA gene was sequenced by using the following oligonucleotide primers (or primers from other conserved regions of the 16S rRNA gene) to amplify the region of interest:

- forward oligonucleotide primer (8F, 5‘ AGAGTTTGATCMTGGCTCAG 3'), and
- reverse oligonucleotide primer (1492R, 5‘ GGYTACCTTGTTACGACTT 3’).

Amplified 16S (and/or 23S) rRNA samples (~1500 bp) were cloned into the TOPO TA cloning vector, sequenced using M13 forward and reverse primers, and then analyzed by the selected laboratory using the BLASTn program from the NCBI website. A substitute identification program may be utilized provided that similar results can be provided. Bacterial identity was selected from the top 25 BLAST® nucleotide database results with maximum identity match greater than 90%. In the event that cloning the 1500 bp fragment into TOPO TA is

unsuccessful, a smaller PCR may be closed using primers in alternate conserved regions of the rRNA gene [14]. If complete single coverage of the 16S rRNA gene was not complete using M13 primers, the portion of high-quality sequence that was obtained was used for BLAST® searches.

5.5 Results from Positive and Negative Control Samples

All the positive controls came back positive. All the negative controls were negative (there was only one, and it was the distilled water). All of the field blanks came back negative except for three. The following samples failed the internal positive control and had significant amounts of growth: IRP-WIPE-10-21-11-ABC-0042; IRP-WIPE-10-21-11-ABC-0047; and IRP-WIPE-10-21-11-ABC-055.

There was not a clear answer as to why these three field blanks had growth. The laboratory performed extractions on blank matrix and spiked matrix concurrently with the samples processed for the study—the gauze spiked with DNA showed variable inhibition (See Table 20). One gauze PC was processed neat, one at 1:5, and three after Qiagen extraction and 1:5 dilution. This observation suggests that the gauze matrix itself may be inhibitory (because the DNA was spiked in 1X TE, which is generally NOT inhibitory). Those three wipes combined with the wetting solution used to process field blanks were even more inhibitory to PCR—possibly the wetting agent released those inhibitory properties from the gauze prior to extraction, which may have exacerbated the problem (particularly as there was no 'sample' to act upon, and all enzymatic and physical actions were applied directly to the gauze itself, rather than to cells and other cellular debris).

Table 20. Results of *Bg* qPCR Analyses of Positive Controls

Sample ID	Dilution	Ct Value	GC/5 mL*	Result**
Filter PC 1	1:5	35.49	204.86	Positive
		35.09	272.53	
Filter PC 2	1:5	31.62	4821.90	Positive
		31.06	6351.25	
Gauze PC 1	1:5	32.65	1510.49	Positive
		32.64	1523.35	
Gauze PC 2	Neat	30.21	8087.46	Positive
		30.04	8778.85	
Gauze PC 3	Qiagen 1:5	32.21	3018.52	Positive
		32.65	2425.59	
Gauze PC 4	Qiagen 1:5	35.29	797.78	Positive
		34.91	963.46	
Gauze PC 5	Qiagen 1:5	38.43	171.44	Positive
		35.78	628.65	
Water PC 1	1:5	31.37	3712.34	Positive
		31.69	2976.29	
Grease PC 1	Neat	32.93	1244.29	Positive
		32.98	1200.10	

* - Gene copies per 5 mL of PCR reaction (after sample extraction, concentration by alcohol precipitation, re-suspension, etc.)

** - Positive = > LOQ was 92.1 GC/5 mL; samples with mean <1 GC/5 mL are considered Negative; samples with multicomponent trace showing no amplification are considered MC Negative.

6. CONCLUSIONS

A study was conducted to evaluate cleanup of a rendering plant after its use for disposal in response to an FAD outbreak. The intent of this study was to develop recommended procedures that could be used to aid in returning a rendering plant to normal operation after use in support of an actual FAD incident.

This effort attempted to achieve three objectives:

- To generate data on fugitive emissions of a biological surrogate during the rendering process;
- To determine the effectiveness of existing plant cleaning procedures for reducing the levels of surrogate on the inside surfaces of the rendering facility; and
- To provide information that could be used to develop standard procedures for appropriately clearing a rendering facility that had been used for "disposal rendering" after an FAD outbreak, as part of returning the rendering facility back to its normal production use.

The Test Team conducted several sampling events at the Darling International (Darling) Rendering Plant located in Des Moines, Iowa, which included:

- Acquiring a series of opportunistic swab samples at the first plant visit to gain an initial insight into the culturable bacterial flora present in the plant;
- Acquiring a series of wipe samples at various locations in the plant to get a more detailed evaluation of background culturable bacterial flora present in the plant;
- Performing an initial sampling effort to focus on potential biological surrogates to use for the Cleaning/Inoculation study;
- Performing a series of laboratory spike tests involving potential biological surrogates in idealized rendering plant sampling matrices and sampling media for air and wipe samples. Based on the results of this and all previous testing, biological and nonbiological surrogates were selected for the Cleaning/Inoculation study; and
- Performing a Cleaning/Inoculation study at the rendering plant to evaluate the movement of the surrogates within the rendering process and subsequent plant cleaning activities.

Initially a thermophilic bacterium such as *G. stearothermophilus* was desired for use as the biological surrogate because the analytical procedures for culturing *G. stearothermophilus* are at temperatures that would inhibit the growth of most other bacterial species in the samples. However, the results from the initial opportunistic samples and the background sampling activities indicated that the PCR procedures used for sequencing the potential surrogate(s) were not sensitive enough to identify *G. stearothermophilus* in the sampling matrices of interest. Only 37.5% of the positive controls (using typical rendering plant matrices) were identified successfully as *G. stearothermophilus* by the procedure. Literature articles [6] further validated the results and indicated that further work on *G. stearothermophilus* may require construction of GEOBAC primers, which was beyond the scope of this study.

Based on those results, a series of challenge samples was evaluated in a bench-scale study using *Bg* and PLGA fluorescent microspheres as potential surrogates in idealized rendering matrix materials (i.e., suet, grease, and DI water). Based on these challenge samples, the inoculum that was selected for the Cleaning/Inoculation study was a mixture of 1E9 CFU of *Bg* spores and 1.47E9 beads of PLGA, with an additional surfactant per gallon of inoculum to prevent clumping of the *Bg s*pores.

Over a series of weekends, the rendering plant was cleaned using cleaning methods normally

utilized by the plant. Following the plant pre-cleaning, a Cleaning/Inoculation study was then conducted in October of 2011. The following conclusions were drawn from the Cleaning/Inoculation study:

- The results of the Cleaning/Inoculation study indicated that no *Bg* DNA was detected in any of the Post-Inoculation- or Post-Cleaning sample extracts from the surface wipes or from the air samples using various PCR techniques. A significant amount of additional effort was devoted to extracting *Bg* DNA from the samples, including sacrificing all of the archived samples in an attempt to use alternate means to amplify the *Bg* DNA and achieve detection. This additional effort was unsuccessful. Although *Bg* was possibly present in low concentrations and below the limit of detection by quantitative PCR (qPCR), nondetection by qPCR was very possibly due to inhibitors present in the sample matrices that carried over during the extraction process. This hypothesis was formulated because putative *Bg* was recoverable on brain heart infusion agar (BHIA) using nonmolecular microbiological techniques and because *Bg* DNA could be extracted from, and detected in, spiked positive controls of pristine gauze and filter matrices, as well as idealized materials similar to rendering plant sample matrices (i.e., suet, grease, and DI water).
- Due to problems with extracting the PLGA microspheres from the sample matrix (both gauze wipes and air filters), PLGA might not be a suitable synthetic surrogate, as the microspheres become permanently immobilized in these sampling matrices. Extraction processes were ineffective at removing PLGA microspheres for quantitation by fluorometer. In addition, autofluorescence from the rendering plant sample matrices (e.g., grease, flesh, bone materials) complicated detection of PLGA microspheres via direct microscopic observation. Other PLGA microspheres with different colors that may not autofluoresce at the same wavelength as the rendering sample matrices may be available. There were two issues with the PLGA microspheres: immobilization on sampling materials and detection interference caused by rendering materials. Other sampling matrices may possibly yield better results with PLGA microspheres.
- Both PLGA and PCR analysis of rendering matrices proved to be difficult. Strides were certainly made to help identify which analysis methods might work better to overcome interferences such as hair, grease, and bone fragments. However, questions linger about qualitative and quantitative analysis of rendering plant samples in the future. In addition, this study raised questions concerning identification and use of a suitable surrogate and the materials that would be necessary to acquire and analyze samples from an environment containing considerable background biological microbes.
- Using nonmolecular microbiological culture techniques, viable bacteria very similar to the *Bg* positive control colony morphology were recovered from eleven of the test sample extracts (five contained putative *Bg* in quantities greater than the LOQ).
- Based on results obtained from nonmolecular biological culture techniques, routine plant cleaning procedures may potentially result in an approximately 1-log reduction in pathogen loading within the potentially contaminated areas of the plant. This result is consistent with results from previous systematic studies examining the effectiveness of different steps of a multi-step cleaning/disinfection process that showed a 1-4 log reduction from individual cleaning/disinfection steps. The plant cleaning procedures used in this study utilized hot water and steam, a combination that would have been expected to remove contamination from surfaces and transfer any removed contamination into the rinse water going into the drains, as opposed to actually killing any surrogate organisms that would have existed in the rinsate. Hot water would not have killed the surrogate spores used in these tests, but may possibly kill some FAD viral agents.

- The cleaning process using the steam and hot water also has the potential to spread the contaminant throughout the plant, even if the cleaning process results in a reduction in the overall levels of contamination. The spread of contamination may be the result of plant personnel tracking the surrogate to various locations within the plant or may be due to aerosol transport. High pressure spraying operations have indeed been shown to result in aerosol transport of spores [13]. However, no air samples exhibited any *Bg* either through PCR analysis or examination of colony morphology.

This study highlights the need for analytical methods that are compatible with the matrices found in rendering facilities.

7. RECOMMENDATIONS

7.1 Recommendations for Future Rendering Plant Sampling/Analytical Efforts

The information that was obtained from this study leads to many questions about the sampling and analysis of the rendering plant matrices and air samples. The study revealed that more work should be done to determine how to sample in a rendering facility environment and to analyze the resulting extracts.

- Both wipe samples and swabs were used for sampling in this study because of the harsh environment (i.e., rough, grimy surfaces) of a rendering plant. Swab samples were negatively impacted by the rough surfaces in a rendering environment, and a single large particle could potentially bias a swab sample. While wipe samples certainly could collect more material, the amount of material collected by a wipe could require multiple dilutions during the biological analysis portion of the study. Also, the materials used in wipe samples interfered with the identification of the PLGA microspheres; i.e., PLGA microspheres became permanently immobilized in the sampling matrices.
- Sample dilution might be a better alternative for these sample matrices or a more desirable solution for the end users, but testing would be necessary to determine the optimal dilution to overcome PCR inhibition without significant loss of target DNA. However, dilution comes at the expense of sensitivity; it is not clear whether a different/additional purification step would be more advantageous than dilution of the inhibitor.
- Newer DNA extraction methods that have been developed recently [15, 16] have shown promise in the ability to extract DNA from complex matrices and may be useful to test on rendering samples.
- Due to the difficulty of extracting *Bg* DNA from the sample matrices, coupled with the success of using nonmolecular microbiological techniques to identify putative *Bg* colonies in heat-shocked samples, the initial desire for a thermophilic bacterium (e.g., *G. stearothermophilus*) to use as a potential biological surrogate for rendering plant studies should be revisited. Results of this study as well as a subsequent literature review [6] indicated that further work on *G. stearothermophilus* may require construction of GEOBAC primers specific to the *Geobacillus* genus based on internal transcribed spacer (ITS) sequences.
- Bench scale recovery tests (for biologicals, both CFU and DNA) using actual rendering plant matrices (instead of idealized matrices) should be conducted to optimize recovery, minimize interferences, and determine suitable surrogates for use in a rendering plant. Similar tests should be conducted for nonbiological surrogates.
- Given that many FADs of interest are viral in nature, development of methods to extract virions and viral DNA from rendering plant matrices may be necessary to show that there is no residual viral loading in the plant following cleaning procedures, or at least that viral loading is below levels pre-determined by the Incident Commander.
- The results of the analyses indicated that PLGA microspheres may not be a suitable synthetic surrogate. The microspheres appear to become immobilized in the sampling matrices, and the particles autofluoresce at a wavelength similar to hair and bone fragments. This behavior makes it difficult to distinguish the PLGA spheres from background. Also, the extraction processes were ineffective at removing PLGA microspheres for quantitation by fluorometer, and autofluorescence from the sample matrices complicated detection of PLGA microspheres via direct microscopic observation. Other variants of the PLGA microspheres may exist that neither autofluoresce at the same

wavelengths as the sampling matrices nor become immobilized in the wipe gauze or air filter materials.

Based on the results of the sampling and methods development work that has been done, an ideal surrogate for use in the field test does not appear to exist. Tradeoffs must be taken into account and a balance struck to pick the best available surrogate given the amount of information that is currently available.

7.2 Recommendations for Developing Plant Cleaning Procedures Following Use of the Plant for Disposal Rendering as Part of an FAD Response

The results from this study suggest that the development of standard operating guidelines to address the cleaning of a rendering plant following its use for disposal rendering as part of an FAD response would include several distinct steps, with precautions being taken to minimize movement of contamination. Due to the size of a typical rendering plant, the diversity of process equipment in the plant, and the level of dirt and grime on many plant surfaces, it is unlikely that fumigation would be recommended for the plant decontamination without first doing extensive testing to verify decontamination efficacy and to assess potential equipment damage. Procedures including surface cleaning and subsequent disinfection may, therefore, be the most appropriate means to restore a rendering plant to normal operation following its use in an FAD response.

The purpose of this study was not specifically to develop the cleaning guidelines, but to develop information that could be used by the rendering industry and agricultural emergency response authorities to develop guidelines that could be used to restore a rendering plant to normal operation following its use in an FAD response.

The following suggestions are offered for inclusion in plant cleaning guidelines:

- Due to the size and diversity of materials of construction in and around the rendering plant and its various process units, as well as the nature of plant operations, there are abundant opportunities to result in the buildup of a potentially significant quantity of dirt, grime, grease, and organic matter on many plant surfaces. This buildup is likely to occur over a period of time significantly longer than the time that the plant would likely be used for disposal rendering. This prior cleaning may present a logistical challenge due to the lead time associated with bringing in a commercial cleaning operation. However, removal of accumulated grime, dirt, and organic matter prior to potentially contaminating the plant with an FAD pathogen may greatly simplify later cleaning and decontamination operations.
- Due to the potential for transport of contamination throughout the plant due to activity of the plant personnel, establishing contaminant control procedures for plant workers prior to delivery of any contaminated materials to the plant may be very important. Contaminant control procedures may include such considerations as:
    - Establishing egress pathways for workers to pass from areas of lower likelihood of contamination to areas of higher likelihood of contamination;
    - Dividing work duties and shift schedules so that workers performing activities in areas of lower likelihood of contamination do not enter areas of higher likelihood of contamination;
    - Establishing procedures for donning and doffing clothing and PPE to minimize contaminant spread; and
    - Using aerosol containment equipment (e.g., tent) at the grinding operation where the most post-inoculation putative positive surrogate samples were observed.

- Due to the potential for cleaning operations to spread contamination around the plant to areas that may previously not have been contaminated, a multi-step (possibly three distinct steps) cleaning/decontamination process, done in a systematic approach with runoff control, appears to be the most effective way to clean the plant for restoration to normal operation. Initial cleaning steps may include such activities as low pressure washing, steam cleaning, and brushing. Minimization of the use of high pressure washing may minimize aerosol transport of potential contaminants.
- The multi-step cleaning/decontamination process might be a three-step process that starts with cleaning only the potentially most heavily contaminated portions of the plant rather than the entire plant. This initial cleaning might focus on removal of organic matter, particularly on the tipping floor, in the feed hopper, the grinder, and on the auger ramps that lead into the cooker, along with the walls and floors in those areas of the plant. This initial cleaning should be staged to move the potentially contaminated materials eventually into the cooker or the drains, such as by cleaning in the following sequence:
    - Tipping floor area walls;
    - Tipping floor;
    - Feed hopper;
    - Grinder; and
    - Augers and ramps.
- During this initial cleaning operation, plant personnel movement from the areas being cleaned to other plant areas that may not be as contaminated should be minimized.
- Utilizing the cooker where possible to process potentially contaminated materials may minimize further contamination of the areas outside the plant.
- Where the cooker cannot be used to process potentially contaminated materials, the remainder may be diverted into the drains, so that runoff can be collected and treated separately.
- Once the heaviest loading of organic matter has been removed from the surfaces in the areas of the plant that have the highest likelihood of contamination (i.e., tipping floor, grinder, feed augers), subsequent cleaning operations should be initiated. These subsequent cleaning steps may include a second pass through the entire plant using steam, detergents, and low pressure spraying of water, with special attention being given to the drain areas, where rendering material may accumulate. A final cleaning step that involves the use of disinfectants that have been registered for use with the FAD organism of interest would then be performed.
- Water and other runoff that is collected in the drains should be treated to kill the FAD pathogen prior to discharge. This step is likely to vary significantly from rendering plant to rendering plant and may require concurrence by permitting authorities who regulate water discharges from the plant.

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APPENDICES

APPENDIX A
CLEMSON DATA REPORT

RESULTS
Study of Fugitive Emissions of a Biological Surrogate Released During the Rendering Process: Pre-Sampling of a Rendering Plant to Select an Appropriate Surrogate Organism

conducted by:

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Clemson University Animal Co-Products Research & Education Center (ACREC)

in response to:

REQUEST FOR QUOTATION FOR
Dynamac Corporation
September 21, 2010
DATS CONTRACT
ANALYTICAL SERVICES

to conduct laboratory services as specified in:

Quality Assurance Project Plan (QAPP) for the Study of Fugitive Emissions Of a Biological Surrogate Released During the Rendering Process: Pre-sampling of a Rendering Plant to Select an Appropriate Surrogate Organism
QA Category III
July 28, 2010

U.S. ENVIRONMENTAL PROTECTION AGENCY
NATIONAL HOMELAND SECURITY RESEARCH CENTER
& NATIONAL DECONTAMINATION TEAM
DECONTAMINATION ANALYTICAL & TECHNICAL SERVICE (DATS) CONTRACT
CONTRACT NUMBER: EP-W-06-089
TDD No. TO-02-10-03-0033

1. PROJECT OBJECTIVES AND ORGANIZATION

1.1 Project Objectives

Dynamac Corporation under the Decontamination Analytical and Technical Services (DATS) Contract will assist the U.S. EPA National Decontamination Team (NDT) and EPA/ National Homeland Security Research Center (NHSRC) in conducting a study to evaluate fugitive emissions of a biological surrogate released from a rendering process. To prepare for this study, it is necessary to evaluate the background concentrations of the potential surrogate(s) selected in a pre-study sampling event. The biological surrogate or surrogates selected to be evaluated in the fugitive emissions study are to be selected based on the results from this pre-sampling effort.

1.2 Project

A total of twenty four (24) swab and two wastewater samples were collected from 13 areas of the Darling International, Inc. Des Moines, IA plant by Anne Busher and Neil Daniell of Dynamac Corporation. One wastewater source was sampled and various hard surfaces and pieces of equipment throughout the plant were sampled. Two swab samples were collected from adjacent areas at each sample location - one for enumeration and one for thermophilic bacterial identification via PCR/DNA sequencing.

Visual observation of the swabs upon receipt at the laboratory indicated varying degrees of slight discoloration of the swab tips and the foam insert within the transport tube. Slight particulate matter was observed on some swabs. The swabs were not significantly deformed across the entire swab surface; only the tips of the swabs appeared flattened (Figures 1-3).

Figure 1 – Swabs as received.

Figure 2 and 3 – Swabs as received.

Table 1 indicates sampling points and measurements that were determined on each sample.

Table 1 - Summary of samples

Sample Number	General Location Description	Matrix	Measurement	Experimental QC	Total Samples
1a, 1b	Raw receiving floor area #1	Swab of surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
2a, 2b	Raw receiving floor area #2	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
3a, 3b	Pit area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
4a, 4b	Pit Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
5a, 5b	Sump Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
6a, 6b	Raw Material Incline Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	Facility – 24
7a, 7b	Raw Grinder Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
8a, 8b	Tallow Tanks/Dryer Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
9a, 9b	Load Out Screw (North End)	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
10a, 10b	Crax Grinder Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
11a, 11b	Crax Storage Bin Area	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
12a, 12b	Tailgate of Truck in Receiving Bay	Swab of non-porous surfaces	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	
13a, 13b	Wastewater from Raw Pit Sump	Liquid	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	2
14a through 16b	Laboratory Blanks	Agar blank, diluent blank, and swab blank	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	TBD	6
17a, 17b	Field Blank	Swab prepared in field as a sample	PCR/DNA Sequencing (a) & Culture/Enumeration (b)	0	2
18	Pos Control (PCR / DNA sequencing)	Pure culture of *G. stearothermophilus*	PCR/Sequencing	0	1
19	Positive Control (swab spike)	Swab spiked with 1E4 cfu *G. stearothermophilus*	Culture/Enumeration	0	1
20	Positive Control (extraction buffer spike)	Extraction buffer spiked with 1E4 *G. stearothermophilus*	Culture/Enumeration	0	1

Enumeration procedures were conducted in duplicate using dilution rates typically used in previous rendering plant swabbing experiments conducted in the various Clemson University laboratories. Results indicated significantly lower counts than obtained in previous research studies. Because of low thermophilic plate counts obtained in the study, the enumeration procedures were repeated using lower dilutions. Results are reported in Tables 2-6. In addition to standard plate count and thermophilic bacterial counts on BHI at 55°C, sample 6 was also enumerated using TSA at 55°C. The template area was 9.62 cm^2 for all sample areas.

Table 2. Standard Plate Count using phosphate/$MgCl_2$ dilution buffer; incubated at 35°C.

PO4/MgCl-SPC	CFU/sq cm
1b	1.89E+06
1b duplicate	2.41E+06
2b	1.25E+04
2b duplicate	1.46E+04
3b	1.87E+04
3b duplicate	4.16E+03
4b	2.08E+03
4b duplicate	4.16E+03
5b	2.08E+03
5b duplicate	0.00E+00
6b	4.54E+06
6b duplicate	7.19E+06
7b	0.00E+00
7b duplicate	0.00E+00
8b	0.00E+00
8b duplicate	0.00E+00
9b	2.08E+03
9b duplicate	0.00E+00
10b	0.00E+00
10b duplicate	0.00E+00
11b	0.00E+00
11b duplicate	0.00E+00
12b	0.00E+00
12b duplicate	0.00E+00
13b	1.83E+05
13b duplicate	5.00E+05
14b	0.00E+00
14b duplicate	0.00E+00
15b	0.00E+00
15b duplicate	0.00E+00
16b	0.00E+00
16b duplicate	0.00E+00
17b	0.00E+00
17b duplicate	0.00E+00
18b	
18b duplicate	
19b	0.00E+00
19b duplicate	0.00E+00
20b	0.00E+00
20 b duplicate	0.00E+00

Table 3. Standard Plate Count using lecithin dilution buffer; incubated at 35°C.

Lecithin-SPC	CFU/sq cm
1b	3.95E+06
1b duplicate	2.49E+06
2b	2.29E+04
2b duplicate	1.04E+04
3b	1.04E+04
3b duplicate	8.32E+03
4b	2.08E+03
4b duplicate	0.00E+00
5b	0.00E+00
5b duplicate	0.00E+00
6b	9.36E+06
6b duplicate	9.15E+06
7b	0.00E+00
7b duplicate	0.00E+00
8b	0.00E+00
8b duplicate	0.00E+00
9b	0.00E+00
9b duplicate	0.00E+00
10b	0.00E+00
10b duplicate	0.00E+00
11b	0.00E+00
11b duplicate	0.00E+00
12b	0.00E+00
12b duplicate	0.00E+00
13b	6.57E+05
13b duplicate	5.20E+05
14b	0.00E+00
14b duplicate	0.00E+00
15b	0.00E+00
15b duplicate	0.00E+00
16b	0.00E+00
16b duplicate	0.00E+00
17b	0.00E+00
17b duplicate	0.00E+00
18b	
18b duplicate	
19b	1.00E+00
19b duplicate	0.00E+00
20b	4.16E+03
20 b duplicate	2.08E+03

Table 4. Thermophilic Plate Count using standard PO4/$MgCl_2$ buffer and BHI agar: incubated at 55°C; Reps 1 and 2.

PO4/MgCl-BHI	CFU/sq cm	PO4/MgCl-BHI	CFU/sq cm
1b	4.16E+03	**1b**	3.12E+02
1b duplicate	1.66E+04	**1b duplicate**	2.08E+02
2b	0.00E+00	**2b**	1.04E+02
2b duplicate	1.04E+04	**2b duplicate**	2.08E+02
3b	0.00E+00	**3b**	1.04E+02
3b duplicate	4.16E+03	**3b duplicate**	1.04E+02
4b	6.24E+03	**4b**	7.28E+02
4b duplicate	0.00E+00	**4b duplicate**	3.12E+02
5b	0.00E+00	**5b**	0.00E+00
5b duplicate	0.00E+00	**5b duplicate**	0.00E+00
6b	0.00E+00	**6b**	3.12E+02
6b duplicate	0.00E+00	**6b duplicate**	5.20E+02
7b	2.08E+03	**7b**	0.00E+00
7b duplicate	0.00E+00	**7b duplicate**	1.04E+02
8b	0.00E+00	**8b**	0.00E+00
8b duplicate	0.00E+00	**8b duplicate**	0.00E+00
9b	0.00E+00	**9b**	0.00E+00
9b duplicate	0.00E+00	**9b duplicate**	0.00E+00
10b	0.00E+00	**10b**	1.04E+02
10b duplicate	0.00E+00	**10b duplicate**	0.00E+00
11b	0.00E+00	**11b**	4.37E+03
11b duplicate	0.00E+00	**11b duplicate**	3.43E+03
12b	0.00E+00	**12b**	0.00E+00
12b duplicate	0.00E+00	**12b duplicate**	0.00E+00
13b	0.00E+00	**13b**	4.16E+02
13b duplicate	0.00E+00	**13b duplicate**	2.08E+02
14b	0.00E+00	**14b**	0.00E+00
14b duplicate	0.00E+00	**14b duplicate**	0.00E+00
15b	0.00E+00	**15b**	0.00E+00
15b duplicate	0.00E+00	**15b duplicate**	0.00E+00
16b	0.00E+00	**16b**	0.00E+00
16b duplicate	0.00E+00	**16b duplicate**	0.00E+00
17b	0.00E+00	**17b**	0.00E+00
17b duplicate	0.00E+00	**17b duplicate**	0.00E+00
18b		**18b**	
18b duplicate		**18b duplicate**	
19b	0.00E+00	**19b**	1.98E+03
19b duplicate	4.16E+03	**19b duplicate**	TNTC
20b	1.01E+04	**20b**	Not measured
20 b duplicate	8.32E+03	**20 b duplicate**	Not measured

Table 5. Thermophilic Plate Count using standard PO4/$MgCl_2$ buffer and TSA on sample 6: incubated at 55°C

PO4/MgCl-TSA	CFU/sq cm
1b	
1b duplicate	
2b	
2b duplicate	
3b	
3b duplicate	
4b	
4b duplicate	
5b	
5b duplicate	
6b	0.00E+00
6b duplicate	0.00E+00
7b	
7b duplicate	
8b	
8b duplicate	
9b	
9b duplicate	
10b	
10b duplicate	
11b	
11b duplicate	
12b	
12b duplicate	
13b	
13b duplicate	
14b	
14b duplicate	
15b	
15b duplicate	
16b	
16b duplicate	
17b	
17b duplicate	
18b	
18b duplicate	
19b	
19b duplicate	
20b	
20 b duplicate	

Table 6. Thermophilic Plate Count using lecithin buffer & BHI: incubated at 55°C; Rep 1 and 2

Lecithin-BHI	CFU/sq cm	Lecithin-BHI	CFU/sq cm
1b	8.32E+03	1b	0.00E+00
1b duplicate	4.16E+03	1b duplicate	1.04E+02
2b	0.00E+00	2b	2.08E+02
2b duplicate	1.04E+04	2b duplicate	2.08E+02
3b	1.25E+04	3b	0.00E+00
3b duplicate	0.00E+00	3b duplicate	1.04E+02
4b	4.16E+03	4b	0.00E+00
4b duplicate	2.08E+03	4b duplicate	2.08E+02
5b	0.00E+00	5b	2.08E+02
5b duplicate	4.16E+03	5b duplicate	4.16E+02
6b	0.00E+00	6b	6.24E+02
6b duplicate	4.16E+03	6b duplicate	8.32E+02
7b	0.00E+00	7b	1.04E+03
7b duplicate	4.16E+03	7b duplicate	1.46E+03
8b	6.24E+03	8b	7.28E+02
8b duplicate	0.00E+00	8b duplicate	5.20E+02
9b	0.00E+00	9b	0.00E+00
9b duplicate	0.00E+00	9b duplicate	0.00E+00
10b	4.16E+03	10b	0.00E+00
10b duplicate	2.08E+03	10b duplicate	0.00E+00
11b	0.00E+00	11b	0.00E+00
11b duplicate	0.00E+00	11b duplicate	0.00E+00
12b	0.00E+00	12b	0.00E+00
12b duplicate	0.00E+00	12b duplicate	0.00E+00
13b	0.00E+00	13b	1.66E+03
13b duplicate	0.00E+00	13b duplicate	1.14E+03
14b	0.00E+00	14b	0.00E+00
14b duplicate	0.00E+00	14b duplicate	0.00E+00
15b	0.00E+00	15b	0.00E+00
15b duplicate	0.00E+00	15b duplicate	0.00E+00
16b	0.00E+00	16b	0.00E+00
16b duplicate	0.00E+00	16b duplicate	0.00E+00
17b	0.00E+00	17b	0.00E+00
17b duplicate	0.00E+00	17b duplicate	0.00E+00
18b		18b	
18b duplicate		18b duplicate	
19b	2.08E+03	19b	7.90E+03
19b duplicate	2.08E+03	19b duplicate	8.11E+03
20b	4.16E+03	20b	Not measured
20 b duplicate	2.08E+03	20 b duplicate	Not measured

Because thermophilic bacterial enumeration results revealed wide variability between duplicates, the experimental procedure on swab samples using BHI and both standard $PO_4/MgCl_2$ and lecithin buffer was repeated twice. Such variability in results has been noted in previous studies on rendering materials.

The second swab was used for identifying thermophilic bacterial strains from the samples. Swabs were pre-enriched with BHI broth overnight at 55°C. The pre-enrichment broth cultures were streaked for isolation on BHI agar and incubated overnight at 55°C. Pure cultures were isolated from the streak plates and inoculated on BHI agar slants which will be incubated at 55°C. Results indicated few isolates obtained from the swab samples. The initial round of plating swabs was conducted in duplicate and results indicated very little growth from the swabs. Therefore, in order to ensure isolates for study, the pre-enriched swabs were plated 10 times each to try to obtain thermophilic isolates. Isolates were transferred to slants and Gram stained.

Colony polymerase chain reaction (PCR) was conducted on the isolates from the slants and the wastewater to amplify the 16S rRNA gene from the bacterial isolates using the:

- forward oligonucleotide primer (8F, 5‘ AGAGTTTGATCMTGGCTCAG 3'), and
- the reverse oligonucleotide primer (1492R, 5‘ GGYTACCTTGTTACGACTT 3’).

Amplified 16S rRNA samples will be sequenced and then analyzed using the BLASTn program on the National Center for Bioinformatics (NCBI) website. Bacterial identity was selected from the top twenty five BLAST nucleotide database results with max identity greater than 90%.

In the initial experiment only 14 isolates successfully amplified and submitted for sequencing. Results of this set of isolates were as follows:

1	Bacillus licheniformis 90%
2	Bacillus licheniformis 81%
3	Bacillus licheniformis 88%
4	No result returned
5	No result returned
6	No result returned
7	No result returned
8	No result returned
9	No result returned
10	No result returned
11	Tepidiphilus sp. or Petrobacter sp. 83%
12	Tepidiphilus margaritifer 99%
13	Aneurinibacillus thermoaerophilus 91%
14	Aneurinibacillus thermoaerophilus 91%

In the second isolation attempt, 72 isolates were obtained. Many of these were deemed likely duplicates based on Gram stain and morphology. After amplifying, these 72 isolates were submitted along with 4 positive controls in duplicate (8 in total). The positive controls were ATCC 7953 *Geobacillus stearothermophilus*, ATCC 12980 *Geobacillus stearothermophilus*, ATCC 12978 *Geobacillus stearothermophilus*, and SPORTROL* Spore Suspensions, NAMSA (VWR Scientific Products, Inc., # 19872-024). Results of this set of isolates were as follows:

1	No result returned
2	Geobacillus stearothermophilus 77%
3	No result returned
4	No result returned
5	Geobacillus sp. 96% or Geobacillus pallidus 94%
6	Bacillus coagulans 97%
7*	No result returned
8*	Geobacillus stearothermophilus 92%
9†	No result returned
10†	No result returned
11§	Geobacillus stearothermophilus 97%
12§	No result returned
13‡	No result returned
14‡	Geobacillus stearothermophilus 97%
15	Klebsiella sp. 99%
16	No result returned
17	Bacillus coagulans 97%
18	Geobacillus pallidus 99%
19	Klebsiella sp 97%
20	No result returned
21	No result returned
22	No result returned
23	No result returned
24	Tepidiphilus sp. or Petrobacter sp. 94%
25	Bacillus thermoamylovorans 94%
26	Bacillus sp. 97%
27	No result returned
28	No result returned
29	No result returned
30	No result returned
31	Klebsiella pneumonia 93%
32	No result returned
33	No result returned
34	No result returned
35	No result returned
36	No result returned
37	No result returned
38	No result returned
39	No result returned
40	No result returned
41	No result returned
42	No result returned
43	No result returned
44	No result returned

45 Aneurinibacillus thermoaerophilus 96%
46 No result returned
47 No result returned
48 No result returned
49 No result returned
50 No result returned
51 No result returned
52 No result returned
53 No result returned
54 No result returned
55 No result returned
56 No result returned
57 No result returned
58 Bacillus licheniformis 94%
59 Klebsiella pneumonia 93%
60 No result returned
61 No result returned
62 No result returned
63 Bacillus licheniformis 77%
64 No result returned
65 No result returned
66 No result returned
67 No result returned
68 No result returned
69 No result returned
70 No result returned
71 No result returned
72 Bacillus licheniformis 96%
73 Bacillus thermoamylovorans 97%
74 Brevibacillus sp 86%
75 Brevibacillus 84%
76 No result returned
77 Bacillus thermoamylovorans 94%
78 Bacillus sp. 90%
79 Bacillus licheniformis 95%
80 No result returned

* Positive Control = ATCC 7953 Geobacillus stearothermophilus
†Positive Control = ATCC 12980 Geobacillus stearothermophilus
§Positive Control = ATCC 12978 Geobacillus stearothermophilus
‡Positive Control = SPORTROL* Spore Suspensions, NAMSA

Bacterial identification results using PCR and amplicon sequencing indicated lack of sensitivity of the procedure to identification of *Geobacillus stearothermophilus*. Only 37.5% of positive controls were successfully identified as *Geobacillus stearothermophilus* by the procedure. Results of this study as well as a literature review indicated that further work on *Geobacillus stearothermophilus* may require construction of GEOBAC primers specific to the *Geobacillus* genus based on internal transcribed spacer (ITS) sequences (Kuisiene, N., J.Raugalas, M. Stuknyte and D. Chitavichius. 2007. Identification of the genus *Geobacillu*s using genus-specific primers, based on the 16S-23SrRNA gene internal transcribed spacer. FEMS Microbiol Lett 277:165–172.)

Appendix B. Battelle Report

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FINAL REPORT

Study of Fugitive Emissions of a Biological Agent Surrogate Released During the Rendering Process

Dynamac Corporation

July 20, 2012

Battelle Columbus Operations

505 King Avenue

Columbus, Ohio 43201-2696

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EXECUTIVE SUMMARY

This Final Report incorporates data presented in the Interim Report dated January 20, 2012 and data obtained during follow-on microbiological and sequence analyses performed in May, 2012. The objective of this project was to detect and enumerate *Bacillus atrophaeus* and poly(D,L-lactide-co-glycolide) (PLGA) microspheres in gauze wipe and filter samples generated during a spiking and decontamination exercise at a rendering facility. In addition, identification of background microbial flora present in the rendering facility was undertaken using sequence analysis of 16S rRNA genes.

Putative *B. atrophaeus* (e.g. bacterial colonies resembling *B. atrophaeus* positive control colonies (2-3mm, orange, entire, raised, smooth) on BHIA) was recovered from 1 of 60 sample extracts originally tested for viable organisms. It was previously reported that 15 of these samples contained putative *B. atrophaeus*, however, upon reassessment of the data it was determined that the majority of these had been misreported due to misinterpretation of the raw data, and only one sample, IRP-IW-10-20-11-ABA-001 had colony morphologies similar to *B. atrophaeus* (Table A1, Appendix A). *B. atrophaeus* DNA was not detected in any of the test samples due to the presence of compounds in the samples that caused significant inhibition of quantitative polymerase chain reaction (qPCR) analysis. Follow-on microbiological analysis, performed on archived samples from all test locations, showed that ten of the archived gauze samples contained putative *B. atrophaeus* (as defined above by colony morphology). Further attempts to enumerate *B. atrophaeus* from these presumptive positive samples yielded: four samples with putative *B. atrophaeus* at or below 1.73×10^3 colony forming units (CFU)/mL, two samples with putative *B. atrophaeus* detected below the limit of quantitation, and four samples with no detectable *B. atrophaeus*. None of the filter samples contained putative *B. atrophaeus*.

Identification of six cultured isolates was obtained by sequence analysis of 16S rRNA genes. These genera included *Proteus*, *Planomicrobium*, and *Curtobacterium*. Sequence analysis of pooled samples showed that the most prevalent bacteria present in all of the samples are *Pseudomonas* species, other genera included: *Stenotrophomonas, Xanthomonas, Comomonas, Herbaspirilium, Lactobacillus, Acinetobacter,* and *Yersinia.*

PLGA microspheres were detected in only two samples; the microspheres became permanently immobilized in the sample matrices, and autofluorescence from the matrices and particulate matter occluded direct visualization of the microspheres by microscopy.

1.0 BACKGROUND AND ASSUMPTIONS

Dynamac Corporation is assisting the U. S. Environmental Protection Agency (EPA) Consequence Management Advisory Team (CMAT) and EPA National Homeland Security Research Center (NHSRC) to evaluate fugitive emissions of a biological agent surrogate released from a rendering process. The ultimate objective was to develop standard procedures for clearing a rendering facility for normal production after it has been used to process contaminated animals from a foreign animal disease outbreak. In support of this larger program, the current project was developed to evaluate detection and quantification of a spore-forming organism, *Bacillus atrophaeus*, and a synthetic surrogate for bacterial agents, 1 μm poly(D,L-lactide-co-glycolide) fluorescent microspheres (PLGA), in gauze wipe and filter

samples generated during rendering processes. An additional project goal was to identify background microorganisms present in the wipe and filter samples through sequence analysis of 16S rRNA genes. The ability to detect and quantify *B. atrophaeus* and PLGA microspheres in rendering facility samples was dependent on several key assumptions:

B. atrophaeus and PLGA microspheres were spiked at levels high enough to withstand dilution of several orders of magnitude and remain above limits of detection for the various analytical methods employed.

B. atrophaeus and PLGA microspheres would disperse uniformly throughout the animal carcasses, mimicking a disease agent.

Sampling matrices would effectively capture dispersed surrogates, and capture would be reversible (i.e., the surrogates could be liberated from the matrices for analysis).

Sample matrices would not interfere with accurate detection of the surrogates.

This project was accomplished in two phases. Phase 1 consisted of method development for detection of PLGA microspheres, and Phase 2 consisted of sample processing and analysis.

Samples were considered to contain target signatures (i.e., viable *B. atrophaeus* and PLGA microspheres) at trace levels; therefore, measures to prevent cross-contamination between samples and/or inadvertent introduction of target signature into a sample were employed, including: thorough decontamination and pre-swab analysis of sample handling areas prior to study initiation and on each day of extraction; thorough decontamination of hands and equipment between samples; and the addition of positive and negative process controls to monitor effectiveness of trace-handling measures.

2.0 OBJECTIVE

The primary objective of this project was to evaluate surrogate dispersion in a rendering plant during normal operations through detection of:

B. atrophaeus by heterotrophic plate count and qPCR,

PLGA microspheres by direct microscopic observation using a suitable filter set.

An additional project objective was to determine the background microbial flora present in samples from the rendering facility through nucleic acid sequence analysis of amplified 16S rRNA genes; elucidation of the background microflora would better enable selection of additional surrogate bacteria for further study.

3.0 MATERIALS and METHODS

Samples were generated by Dynamac Corporation in late October 2011. A truck load of animal carcasses was spiked with viable *B. atrophaeus* and PLGA microspheres, and carcasses were subsequently handled and processed in a rendering plant according to normal operations. Wipe and air samples were collected before, during, and after processing and shipped to Battelle for analysis. Samples were received cold (wet ice) in two separate shipments; each was logged and returned to cold storage at 2-8°C until processed. All processing areas, including the biological safety cabinet and incubator, were thoroughly decontaminated and swabs were taken and plated onto brain heart infusion agar (BHIA) to ensure that working areas were sterile prior to sample processing. An additional swab was taken and plated on BHIA on each day of sample extractions to serve as a laboratory blank for verification that the working surface was free of *B. atrophaeus* each day of extraction. Sample processing occurred in five batches over the course of three weeks, and analytical positive and negative controls were created for each batch as follows: a single negative control and a single positive control for each matrix type was extracted in the batch. Negative controls (Matrix Blank 1, 2, etc.) comprised a single pristine matrix, while positive controls (Matrix PC 1, 2 etc.) comprised a single pristine matrix spiked with *B. atrophaeus* genomic DNA (gDNA) at 1 x 10^7 gene copies (GC)/sample and PLGA microspheres at 0.05 mg/sample. Control matrices were provided by Dynamac and were identical to sample matrices. Controls were processed in tandem with the samples, and each received identical treatment to the sample matrices.

Each sample or control was extracted according to a project-specific work instruction (DWI-01, Work Instructions for the Extraction of Microorganisms, Nucleic Acids, and PLGA Microspheres from Environmental Samples) provided in Appendix C. Briefly, samples were removed from their original containers and placed into sterile 250 mL bottles and phosphate buffered saline (PBS), Teknova, Hollister, CA (Catalog Number PA205) was added (12 or 15 mL for filter and gauze wipe samples, respectively). Each sample was mixed by vortexing (approximately 30 seconds) and incubated for 30 minutes at room temperature. At this point, 1 mL was removed to serve as the microbiology extract, and the remaining sample was extracted for nucleic acids (DNA). Microbiology extracts from extraction sets 1 and 2 (60 total samples) were plated onto BHIA (200 µL per plate) and incubated at 36 ± 2°C overnight to isolate single colonies of bacteria; the remaining microbiology extracts were stored at 4°C until being further processed for isolation of bacterial DNA. Briefly, 1 µL herring sperm carrier DNA (hsDNA), Promega (Catalog Number)/mL sample and 1% (v:v) sodium dodecyl sulfate (SDS), Fisher BioReagents, Pittsburgh, PA (Catalog Number BP1311-200) were added to the remaining volume of extracts and samples were incubated for 30 minutes at 65°C. The original sample matrix (filter or gauze) was preserved in the extraction vessel for detection of PLGA microspheres by microscopic analysis (from method development testing, PLGA microspheres spiked onto gauze and filter matrices could not be detected in aqueous solution, but could be visually observed on filters and gauze), and the aqueous extract was transferred to a sterile Oakridge tube. DNA was concentrated using method ABAT-V-012 (Concentration of Nucleic Acids by Isopropanol Precipitation). In this method, DNA was precipitated overnight with isopropanol, recovered by centrifugation, washed with 70% ethanol and resuspended in 1X Tris EDTA (TE) buffer (10 mM Tris and 1 mM EDTA), pH 7, Fisher BioReagents, Pittsburgh, PA (Catalog Number BP2476-1). Pre-amplification analyses of the extracts were not conducted.

3.1 *B. atrophaeus* Detection by Quantitative PCR

For DNA analysis, duplicate 5 µL aliquots of sample extracts were assayed via qPCR using an assay specific for the *rtp* gene of *B. atrophaeus* on an ABI 7900HT platform. The limit of detection (LOD) and limit of quantification (LOQ) for this assay were determined to be 92.1 gene copies (GC)/5 µL. Prior to target analysis, sample extracts were tested for inhibition using the Applied Biosystems (ABI) TaqMan® Exogenous Internal Positive Control Reagents kit according to method ABAT-V-007 (TaqMan Inhibition Analysis on the 7900HT). Neat, 1:5, and 1:10 dilutions of each sample were initially assayed. In the event extracts did not pass internal positive control (IPC) testing at the 1:10 dilutions, they were further purified using a Qiagen (Valencia, CA) QIAQuick PCR Purification kit according to the manufacturer's instructions. The Qiagen-purified sample extracts were further diluted and tested by IPC analysis at Qiagen Neat (QN), Qiagen 1:5 (Q5), Qiagen 1:10 (Q10) and Qiagen 1:20 (Q20) dilutions. Sample extracts that passed IPC were analyzed for *B. atrophaeus* DNA at the highest concentration passing the inhibition test according to method ABAT-V-008 (To Prepare a 96-Well Plate for DNA Quantitation on the 7900HT). The Ct (threshold cycle) value and estimated nucleic acid quantity based on the input standard curve were compiled, along with an amplification plot and a trace of fluorescent signals (multicomponent plot) for each replicate sample. The multicomponent plot was examined for each sample replicate to verify results; positive detections showed elevated signal from the reporter fluorescent molecule (FAM). Assay acceptance criteria included the following:

Valid standard curve with three or more duplicate points and R^2 value of ≥0.95,

No amplification ("Undetermined" at 45 cycles) in No Template Control (NTC) wells.

A small subset of sample extracts IRP-AIR-10-24-11-ABC-018 to IRP-AIR-10-24-11-ABC-025 and sample IRP-AIR-10-24-11-ABC-27 was not analyzed by qPCR. This set of sample extracts was amplified on the ABI 9700 thermocycler using *B. atrophaeus rtp* primers and analyzed by gel electrophoresis, with direct visualization of ethidium bromide-stained target amplicon (*B. atrophaeus rtp*, 82 bp). Positive and negative control reactions were prepared and analyzed along with the sample extracts. Each sample was initially analyzed on a 2% agarose gel with 1X TAE running buffer (10 µL sample per well), and an additional 1.2% gel was run to compare pooled sample extracts (5 µL each) against pooled NTCs and the positive control reaction. Each gel contained an appropriate molecular weight marker, either Quick-Load 2-log ladder (New England Biolabs, Ipswich, MA, 2% gels) or 1Kb Plus Track It Ladder (Life Technologies™, Carlsbad, CA, 1.2% gels).

Sample extracts that did not pass IPC were subject to PCR using the Phire® Animal Tissue Direct PCR Kit (ThermoScientific, Pittsburgh, PA). Phire® PCR was run according to the manufacturer's instructions using pooled DNA extracts. Samples were pooled by combining 3 µL of each extract in groups of nine or ten (Table 1). Reactions were created by combining 5 µL of each pooled sample extract with 25 µL 2X Phire® Animal Tissue PCR Buffer, 10.875 µL RNase-Free water, 2.5 µL of each *B. atrophaeus rtp* forward and reverse primer (10 µM), and 1 µL of Phire® Hot Start II DNA Polymerase. Reactions were processed with the following cycling parameters: initial denaturation (5 minutes, 98°C); 40 cycles of denaturation (98°C, 5 seconds), annealing (65°C, 5 seconds), and extension (72°C, 20 seconds); a final 1 minute extension at 72°C. Each reaction was analyzed on 1.2% agarose gels; 25 µL of

each PCR reaction was combined with 5 µL 6X Track It Loading Dye (Life Technologies™, Carlsbad, CA) and run against the 1Kb Plus Track It Ladder (Invitrogen).

3.2 Detection of PLGA Microspheres

During method development, a 96-well microtiter plate assay was developed for detection of PLGA microspheres (Phosphorex, Inc., St. Fall River, MA), Catalog Number LGFG1000, in an aqueous extract. PLGA microspheres were diluted in 1X PBS to create a 10 mg/mL top concentration, which was then analyzed by dilution to extinction on two platforms: 1) SpectraMax M2 Multi-Mode Microplate Reader, and 2) Victor Fluorometer (0.1 and 1 second exposure times). PLGA microspheres were analyzed in concentrations that ranged from 10 mg/mL to 1.19 x 10^{-6} mg/mL (diluted 1:2 in 1X PBS). The working range of the SpectraMax M2 was determined to be 10 to 0.02 mg/mL whereas the working range of the Victor was 10 to 0.001 mg/mL. Due to the lower limit of detection obtained using the Victor fluorometer, that instrument was chosen for further assay development, and a standard curve was prepared and validated from 10 to 0.001 mg/mL.

Table 1. Pooled Sample Extracts for Phire® Animal Tissue Direct PCR Kit

Pooled Sample	Sample Extracts Combined	Pooled Sample	Sample Extracts Combined
1	IRP-WIPE-10-19-11-ABC-B2 (QN) IRP-WIPE-10-21-11-ABC-0015 (QN) IRP-WIPE-10-21-11-ABC-0016 (QN) IRP-WIPE-10-21-11-ABC-0017 (QN) IRP-WIPE-10-21-11-ABC-0030 (QN) IRP-WIPE-10-21-11-ABC-0032 (QN) IRP-WIPE-10-21-11-ABC-0035 (QN) IRP-WIPE-10-21-11-ABC-0037 (QN) IRP-WIPE-10-21-11-ABC-0042 (QN)	4	IRP-WIPE-10-24-11-ABC-0072 (QN) IRP-WIPE-10-24-11-ABC-0073 (QN) IRP-WIPE-10-24-11-ABC-0074 (QN) IRP-WIPE-10-24-11-ABC-0075 (QN) IRP-WIPE-10-24-11-ABC-0076 (QN) IRP-WIPE-10-24-11-ABC-0077 (QN) IRP-WIPE-10-24-11-ABC-0078 (QN) IRP-WIPE-10-24-11-ABC-0080 (QN) IRP-WIPE-10-24-11-ABC-0081 (QN) IRP-WIPE-10-24-11-ABC-0096 (QN)
2	IRP-WIPE-10-21-11-ABC-0044 (QN) IRP-WIPE-10-21-11-ABC-0047 (QN) IRP-WIPE-10-24-11-ABC-0051 (QN) IRP-WIPE-10-24-11-ABC-0052 (QN) IRP-WIPE-10-24-11-ABC-0055 (QN) IRP-WIPE-10-24-11-ABC-0056 (QN) IRP-WIPE-10-24-11-ABC-0057 (QN) IRP-WIPE-10-24-11-ABC-0058 (QN) IRP-WIPE-10-24-11-ABC-0059 (QN) IRP-WIPE-10-24-11-ABC-0060 (QN)	5	IRP-WIPE-10-24-11-ABC-0083 (QN) IRP-WIPE-10-24-11-ABC-0084 (QN) IRP-WIPE-10-24-11-ABC-0086 (QN) IRP-WIPE-10-24-11-ABC-0087 (QN) IRP-WIPE-10-24-11-ABC-0088 (QN) IRP-WIPE-10-24-11-ABC-0089 (QN) IRP-WIPE-10-24-11-ABC-0090 (QN) IRP-WIPE-10-24-11-ABC-0092 (QN) IRP-WIPE-10-24-11-ABC-0093 (QN)
3	IRP-WIPE-10-24-11-ABC-0061 (QN) IRP-WIPE-10-24-11-ABC-0062 (QN) IRP-WIPE-10-24-11-ABC-0064 (QN) IRP-WIPE-10-24-11-ABC-0065 (QN) IRP-WIPE-10-24-11-ABC-0066 (QN) IRP-WIPE-10-24-11-ABC-0067 (QN)		

	IRP-WIPE-10-24-11-ABC-0068 (QN) IRP-WIPE-10-24-11-ABC-0070 (QN) IRP-WIPE-10-24-11-ABC-0071 (QN) IRP-WIPE-10-20-11-ABC-001 (QN)

Once the assay was established, verification of the proposed extraction method was initiated. Control sample matrices (gauze and filters) were spiked with 1 mg PLGA microspheres, and a mock extraction was performed according to DWI-01; it was anticipated that

the PLGA microspheres would be removed from the gauze and filter matrices and suspended in the extract, whereupon they would be recovered during the final filtration. However, it was discovered that the PLGA microspheres adsorbed to the gauze and filter matrices, and all attempts to remove them were unsuccessful. At the advice of the PLGA microsphere manufacturer, Phosphorex, Inc., 25 mL of a 2.5% solution of polyvinyl alcohol (PVA) was added to each spiked filter and gauze sample, followed by vortex agitation for 1 minute. Room temperature incubation was continued up to 30 minutes with intermittent agitation by vortex. As no change was observed after 30 minutes, a waterbath sonicator was used to agitate each sample for 5 minutes. Even after sonication in PVA, deposits of PLGA microspheres, visible to the naked eye, remained on both types of sample.

Due to the apparent irreversible immobilization of PLGA microspheres onto the filter and gauze sample matrices, qualitative detection/non-detection of PLGA microspheres was accomplished by direct microscopy using a Zeiss Axioscope epifluorescent microscope equipped with a filter set with excitation at 495 and emission at 517 nm. Representative images were captured using a Zeiss color camera.

3.3 Enumeration of Putative Viable *B. atrophaeus* in Archived Samples

Original filter samples and archived gauze wipe samples were extracted according to the work instruction DWI-01. Samples were pre-wet with 1X PBS extraction buffer (2 mL for filter samples, 5 mL for gauze samples) and mixed by vortexing for 30 seconds. An additional 10 mL of 1X PBS was added to each sample, and samples were incubated at room temperature (25±3°C) for 30 minutes. Samples were mixed by vortexing at 0, 15, and 30 minutes. Following incubation, 200 µL of each sample was spread-plated onto brain heart infusion agar (BHIA) and incubated overnight at 30°C. Plates were observed for microbiological growth, and morphologies of resultant colonies were compared to that of an overnight positive control of *B. atrophaeus* plated onto BHIA. Any samples containing putative *B. atrophaeus* were re-plated onto fresh BHIA for enumeration. The putative *B. atrophaeus* samples were diluted in 1X PBS and heat-shocked by incubation at 65°C for 30 minutes to kill any vegetative cells that might out-compete the spore-forming *B. atrophaeus*. Positive and negative controls were processed along with the samples to ensure process efficacy. Negative controls were prepared by transferring clean filter and gauze matrices into sterile sample reservoirs; positive controls were prepared by transferring clean filters and gauze matrices into sterile sample reservoirs and spiking with an aliquot of *B. atrophaeus*.

3.4 Identification of Background Microflora by Sequence Analysis

3.4.1 Selection of Unknown Isolates and Pooled Samples

Microorganisms recovered on BHIA from the 60 original analyzed wipe and filter samples were selected for follow-on analysis by 16S rRNA sequencing. Thirty isolates that did not have similar morphology to *B. atrophaeus* were selected and streaked for isolation on BHIA, followed by incubation for 16 – 48 hours at 36 ± 2°C. Appendix A lists the isolate morphology and the sample from which the isolate originated. *B. atrophaeus* ATCC 9372 was included as a positive control. A portion of the samples from each of the nucleic acid extract batches were combined to generate five pooled samples for metagenomic 16S rRNA analysis using the Ion Torrent™ Personal Genome Machine™ (PGM™) Sequencer (Life Technologies).

3.4.2 Extraction of DNA

Three different extraction techniques were used to prepare DNA for 16S rRNA amplification. Initially, each of the 30 isolates and aliquots of the five pooled samples (i.e. the remaining microbiological extracts pooled according to extraction date) were extracted following the DNeasy® Gram-positive bacteria protocol (Qiagen); however, the DNeasy® extracts could not be used directly for PCR due to background 16S rRNA DNA that amplified in the enzymatic lysis buffer, which contained lysozyme, triton X-100, and 1X TE. As neither lysozyme nor triton X-100 is readily-available in a certified DNA-free formulation, a thermolysis technique was attempted to circumvent the need for DNA-free lysis buffer. However, subsequent attempts to amplify the 16S rRNA gene from the five pooled samples were unsuccessful following thermolysis. Finally, a OneStep™ PCR Inhibitor Removal Kit (Zymo) was used on the pooled samples prior to PCR amplification.

3.4.2.1 Extraction using DNeasy® Blood and Tissue Kit. Enzymatic lysis buffer was prepared as follows: 2 mL of Tris-EDTA, 10X (Fisher), 120 µL of Triton X-100 (Fisher), and 2 mL of 100 mg/mL lysozyme, egg white (Fisher) was added to 5.88 mL of MilliQ distilled water. One to several colonies, depending on size, were selected for extraction; after addition of the colonies to a tube containing 180 µL of the above enzymatic lysis buffer, extractions were completed following the manufacturer's instructions for Gram-positive bacteria. To prepare pooled samples for extraction, 1 mL of each pooled sample was centrifuged at 5,000 *x g* for 10 minutes, and the pellet was suspended in 180 µL of enzymatic lysis buffer and extracted according to the manufacturer's instructions as stated above.

3.4.2.2 Extraction via Thermolysis. DNA from the 30 isolated colony morphologies were extracted by adding one to several colonies, depending on size, to a tube containing 250 µL of 1X Tris-EDTA (Fisher). The samples were autoclaved using a liquid cycle for 10 minutes at 121°C. Following autoclave treatment, the samples were cooled to room temperature and stored at -80°C until ready for use. Pooled samples were treated in the same manner, after 10 µL of each pooled sample (described in 3.4.2) was added to a separate tube containing 250 µL of 1X Tris-EDTA.

3.4.2.3 Extraction of PCR inhibitors using OneStep™ column. Fifty microliters of each of the original pooled samples (described in 3.4.2) was processed using the OneStep™ PCR Inhibitor Removal Kit (Zymo) following the manufacturer's instructions.

3.4.3 Amplification of 16S rRNA

The 30 isolated colonies and five pooled samples were subject to PCR using 8F (isolated colonies and pooled samples) or 27F (pooled samples) and 1492R 16S rRNA primers (Table 2).

Table 2. 16S rRNA Primer Sequences[1]

Primer ID	Sequence
8F	5'-AGAGTTTGATCMTGGCTCAG-3'
27F	5,-AGAGTTTGATCCTGGCTCAG -3’
1492R	5'-GGYTACCTTGTTACGACTT-3'

A high-fidelity polymerase, Phusion™ (New England Biolabs, Ipswich, MA) was used to amplify the 16S rRNA gene from each of the 30 isolated colonies. PCR amplification of the 30 isolated colonies was carried out in 50 µL total volumes containing: 1 X Phusion™ HF Buffer, 0.02 U/µL of Phusion™ DNA Polymerase, 0.5 µM of each 16S primer, and 0.2 µM of each dNTP inoculated with 5 µL of thermolysed colonies. Cycling conditions were carried out on a 9700 thermocycler (Applied Biosystems, Carlsbad, CA) according to the following: an initial hold at 98°C for 30 seconds; 35 cycles of denaturation (98°C for 10 seconds), annealing (55°C for 30 seconds), and extension (72°C for 1 minute); a final hold at 72°C for 5 minutes. For samples amplified with primers 27F and 1492R, the annealing temperature was raised to 56°C. PCR products were quantified by UV-absorbance using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific).

Initially, pooled samples were subject to PCR using primers 8F and 1492R, and then amplified using a polymerase with high resistance to many PCR inhibitors, Phire® (New England Biolabs, Ipswich, MA). The Phire® PCR was carried out in 50 µL total volumes containing: 1 X Phire® Animal Tissue PCR Buffer, 1 µL of Phire® Hot Start II DNA Polymerase, and 0.5 µM of each primer, inoculated with 5 µL of OneStep™ cleaned pooled sample. Cycling conditions were carried out on a 9700 thermocycler with an initial hold at 98 °C for 5 minutes; 40 cycles of denaturation (98°C for 5 seconds), annealing (55°C for 5 seconds), and extension (72°C for 40 seconds); a final hold at 72 °C for 1 minute. Following amplification of the 16S rRNA gene, the size of the amplified product was checked using 1.2 % Agarose E-Gel® (Life Technologies) and an E-Gel® 1 Kb Plus DNA ladder (Life Technologies).

A second amplification of the pooled samples was undertaken using the 27F and 1492R primers; no further amplification was required for these PCR products prior to sequencing.

3.4.4 Sequencing of 16S rRNA genes

[1] Baker, G. C., Smith, J. J., Cowan. Review and re-analysis of domain-specific 16S primers. Journal of Micro. Methods, 55 (3): 541-555. 2003.

3.4.4.1 Sequencing of 16S rRNA from Isolated Colonies using Applied Biosystems 3130 Genetic Analyzer. The 16S rRNA PCR products generated from isolated colonies were purified using the GenElute™ PCR Clean-up Kit (Sigma-Aldrich), and the concentration of each PCR product was determined using the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Pittsburgh, PA). Forward and reverse cycle sequencing reactions were set up using the same 8F and 1492R PCR primers that yielded the original PCR product. Cycle sequencing was carried out using BigDye® Terminator v3.1 (Life Technologies™, Carlsbad, CA) in 20 µL total volumes containing: 4 µL of Ready Reaction Mix, 2 µL of BigDye Sequencing Buffer, 5 pmol primer, and 20 – 40 ng of 16S rRNA PCR product. Cycling conditions were carried out on a 9700 thermocycler (Applied Biosystems) with an initial hold at 96°C for 1 minute; 25 cycles of denaturation (96°C for 10 seconds), annealing (50°C for 5 seconds), and extension (60°C for 4 minutes). A positive control, pGEM®-3Zf(+), and NTC negative controls were included. Cycle sequencing reactions were purified using the BigDye® XTerminator™ Purification Kit (Applied Biosystems) following the manufacturer's instructions.

Capillary electrophoresis was run on each purified cycle sequencing reaction using Applied Biosystems 3130 Genetic Analyzer with the parameters shown in Table 3.

Table 3. Applied Biosystems 3130 Genetic Analyzer Parameters

Specific Parameters	
Parameter	**Setting**
Template	BDx_StdSeq50_POP7
Oven Temperature	60 °C
Poly Fill Volume	5020 steps
Current Stability	5.0 Amps
Pre-Run Voltage	15.0 kVolts
Pre-Run Time	180 seconds
Injection Voltage	1.6 kVolts
Injection Time	4 seconds
Voltage Number of Steps	40 nk
Voltage Step Interval	15 seconds
Data Delay Time	480 seconds
Run Voltage	8.5 kVolts

Run Time	6000 seconds

All raw sequencing files were imported into Sequencing Analysis Software v5.2 (Applied Biosystems) and analyzed using the KB™ basecaller to provide per-base quality value predictions.

3.4.4.2 Sequencing of 16S rRNA Amplified with Primers 8F and 1492R using Life Technologies Ion Torrent Personal Genome Machine (PGM™). A total of fourteen 16S rRNA amplicons, including a positive and negative control, 1550 base pairs (bp) in length, were initially processed to create a sequencing library. Library preparation generated a pool of amplicons tagged with a specific molecular barcode that allowed multiplexing of samples for analysis on a single PGM™ semiconductor chip. The Ion DNA Barcoding 1-16 kit (Life Technologies™, Carlsbad, CA) was used to prepare the library for the multiplexing experiment. Briefly, each of the 16S DNA amplicons separately underwent enzymatic shearing to fractionate the 1550 bp products; a purification step was performed using Agencourt® AMPure® magnetic particles (Beckman Coulter, Brea, CA) according to the manufacturer's instructions; and Ion Barcode Adapters™ were ligated to the fragmented, purified DNA. An additional purification step was performed using the Agencourt® AMPure® magnetic particles to remove small molecular weight fragments. Following purification, an additional PCR was performed incorporate unique molecular barcodes onto the adapter-modified, fragmented DNA and further amplify each molecule. After PCR, a final purification step was performed using the Agencourt® AMPure® magnetic particles. Each molecule in the final bar-coded library preparation was approximately 180-210 base pairs in length, including amplicon sequence, adapter, and barcode. Individual reactions were measured using the NanoDrop® (Thermo Scientific, Pittsburgh, PA) to quantify DNA concentrations prior to pooling a portion of each reaction into a single aggregate sample. The concentration of the aggregate sample was measured again using the NanoDrop® to determine the library pool dilution required for sequencing.

To prepare the aggregate, barcoded sample for sequencing, clonal amplification was performed on the Ion OneTouch™ instrument. Briefly, the aggregate, barcoded library was combined with IonSphere Particles™ (ISPs) followed by clonal amplification in an oil emulsion PCR, which binds a single molecule to each particle and creates multiple copies of each particle-bound fragment. Immediately following clonal amplification, the particle-bound fragments were enriched using the Ion OneTouch ES™ instrument; this process removes unbound particles and unbound library fragments to enrich for particle-bound fragments. At this point, a quality control check was performed, whereby a small amount of the enriched ISPs was quantitated using the Qubit® 2.0 Flourometer (Life Technologies™, Carlsbad, CA) to determine the extent of enrichment. After enrichment, ISPs were loaded into an Ion 316™ chip (a single ISP per well) and sequencing was carried out according to manufacturer's instructions.

3.4.4.3 Sequencing of 16S rRNA Amplified with Primers 27F and 1492R using Life Technologies Ion Torrent PGM™. Qualitative and quantitative measurements of 16S amplicons were made using the Qubit dsDNA BR Assay Kit on the Qubit 2.0 fluorometer followed by analysis on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) using DNA High Sensitivity chips. Following this, 16S amplicon samples were fragmented using a Covaris S220 sonicator (Covaris, Inc, Woburn, MA) to generate approximately 300bp fragments. Fragmentation quality was assessed using an

Agilent Bioanalyzer. Sequencing libraries were made using Life Technologies™ Ion Plus Fragment Library kit for 200bp sequencing. Library quality was verified using the Agilent Bioanalyzer and the Qubit. Clonal amplification was performed on an Ion One Touch instrument using the Ion Xpress™ Template 200 Kit (Life Technologies™, Carlsbad, CA). Enrichment for the ISP's was done on the Ion One Touch ES, and quantification of the percent templated ISP's was performed on the Qubit fluorometer. Sequencing was performed with 316 chips on an IonTorrent PGM sequencer using the Ion Sequencing 200 kit. The IonTorrent Suite Server (1.5.1) performed base calling and output raw sequence data in FASTQ format.

3.4.5 Sequence Analysis of 16S rRNA genes

Sequence reads from the ABI 3130 with a length of greater than 200 base pairs and high quality base calls were subject to BLASTn (GenBank, http://blast.ncbi.nlm.nih.gov/), searching against the 16S microbial database. The BLAST nucleotide results with the highest maximum identity percentage were reported.

3.4.5.1 Bioinformatics. FASTQ files were loaded into CLCBio Genomics Workbench software V 4.9. Trimming of sequence reads was performed to remove PCR primer sequences and low quality reads (0.05 quality threshold). A final filtering of reads was performed to select for reads of >175 bps. The NCBI 16S rRNA (v6/15/2102) sequence database was loaded into CLCBio as a reference library. Two bioinformatics analyses were performed. First, read files were processed using the Battelle Galileo high performance compute cluster and the Basic Local Alignment Search Tool (BLAST®) (National Library of Medicine, Bethesda, MD). Reads were searched against the NCBI 16S rRNA gene database (v6/15/2102) (NCBI, Bethesda, MD), which contained entries for 7,545 sequences. Search results were filtered for sequences with ≥97% identity. The output from this search resulted in a list of taxonomic IDs, associated organism names, and number of reads per taxID for each sample. Krona[2] v. 2.1 was used to create a comparative chart for viewing the relative abundance of organisms at the genus level for each sample. A final filtering of results was performed to include only taxa identified by numbers of hits greater than 0.1% (1:1000) of the total representation per sample. The second analysis, the Battelle QUEST™ tool, a recent R&D development using weighted probabilities based on genome coverage from reference aligned data, was used to measure the amount of individual reads mapping to each 16S rRNA sequence with the optimized parameters in CLCBio software and backend statistical analysis. The output was reported as most probable species present in the sample.

4.0 RESULTS AND DISCUSSION

4.1 *B. atrophaeus* Detection by Quantitative PCR

[2] Ondov BD, Bergman NH, and Phillippy AM. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* 2011. 12:385.

Quantitative PCR (qPCR) analysis for *B. atrophaeus* signatures was complicated by the co-extraction of inhibitory components from sample matrices. The extraction method used for this project is a slight modification of a method that has been used by Battelle for over ten years to extract and recover trace nucleic acids from environmental samples. Sample matrices successfully processed using this method include aqueous, soil, cellulose, food, and fabric compositions. Generally, any inhibitory components that are co-extracted with the DNA can be counteracted by dilution (1:5 or 1:10) of the sample extract in 1X TE containing 10 mM Tris and 1 mM EDTA. In this case, only about a third of the sample extracts could be analyzed Neat, 1:5, or 1:10; the remaining two thirds of the sample extracts required further purification using a Qiagen (Valencia, CA) QIAquick PCR purification kit. The Qiagen samples were diluted and tested for inhibition at Neat, 1:5, 1:10, and 1:20.

Sample extracts that passed IPC were analyzed in duplicate for *B. atrophaeus rtp* signatures at the highest concentration that passed IPC. Table 4 shows the results of qPCR, including the analyzed dilution, the threshold cycle (Ct), and quantity in GC/5 µL for positive control samples. *B. atrophaeus* DNA was not detected in any of the sample extracts; sample number IRP-WIPE-10-21-11-ABC-24 was first thought to be positive, but upon further investigation, the multicomponent plot showed that the fluorescent signal in those wells was extremely high, and true amplification did not occur. Samples that were inhibited at all tested dilutions (Neat, 1:5, 1:10, QN, Q5, Q10, and Q20) were subject to PCR using the Phire® Animal

Table 4. Results of *Bacillus atrophaeus* qPCR Analyses

Sample ID	Dilution	Ct Value	GC/5 µL[a]	Result[b]
Filter PC 1	1:5	35.49	204.86	Positive
		35.09	272.53	
Filter PC 2	1:5	31.62	4821.90	Positive
		31.06	6351.25	
Gauze PC 1	1:5	32.65	1510.49	Positive
		32.64	1523.35	
Gauze PC 2	Neat	30.21	8087.46	Positive
		30.04	8778.85	
Gauze PC 3	Qiagen 1:5	32.21	3018.52	Positive
		32.65	2425.59	
Gauze PC 4	Qiagen 1:5	35.29	797.78	Positive
		34.91	963.46	

Sample ID	Dilution	Ct Value	GC/5 µL[a]	Result[b]
Gauze PC 5	Qiagen 1:5	38.43	171.44	Positive
		35.78	628.65	
Water PC 1	1:5	31.37	3712.34	Positive
		31.69	2976.29	
Grease PC 1	Neat	32.93	1244.29	Positive
		32.98	1200.10	

[a] Gene copies per 5 µL of PCR reaction (after sample extraction, concentration by alcohol precipitation, re-suspension, etc.);
[b] Positive = >Limit of Quantitation (LOQ); LOQ was 92.1 GC/5 µL; samples with mean <1 GC/5 µL are considered Negative; samples with multicomponent trace showing no amplification are considered MC Negative.

[c] These sample extracts were inhibited Neat, 1:5, 1:10, and 1:20, they were further purified by Qiagen kit and diluted to overcome inhibition

[d] Multicomponent

Tissue Direct PCR Kit after pooling DNA extracts into five composite samples comprised of nine or ten sample extracts (Table 1). Phire® PCR was unsuccessful at amplification under these conditions; no amplification was observed in any sample, including the positive control (1 x 10^4 GC/5 µL amplified standard). Because the positive control reaction did not amplify, it appears that the PCR conditions were sub-optimal, and it is not possible to determine from this analysis

whether these inhibited samples contain *B. atrophaeus* DNA. The results for a small subset of

samples was inadvertently omitted from the Interim Report, these samples were also analyzed by Phire® Animal Tissue Direct PCR Kit, in duplicate reactions using the 7900HT, rather than in the sample pools as described above. These samples did not amplify, also likely due to inhibition of the Phire® polymerase.

Sample extracts IRP-AIR-10-24-11-ABC-018 to IRP-AIR-10-24-11-ABC-025 and sample IRP-AIR-10-24-11-ABC-27 were amplified on the ABI 9700 instrument using the *B. atrophaeus rtp* primers and analyzed by gel electrophoresis (target amplicon 82 bp). Positive control reactions containing 1000 GC/5 µL standard control material, and negative control reactions (NTC) containing 1X TE, were prepared and analyzed along with the sample extracts. No *B. atrophaeus* DNA was detected in the samples or NTCs, but a band was observed in the 1000 GC/5 µL standard positive control well consistent with the expected 82 bp amplicon (Figure 1).

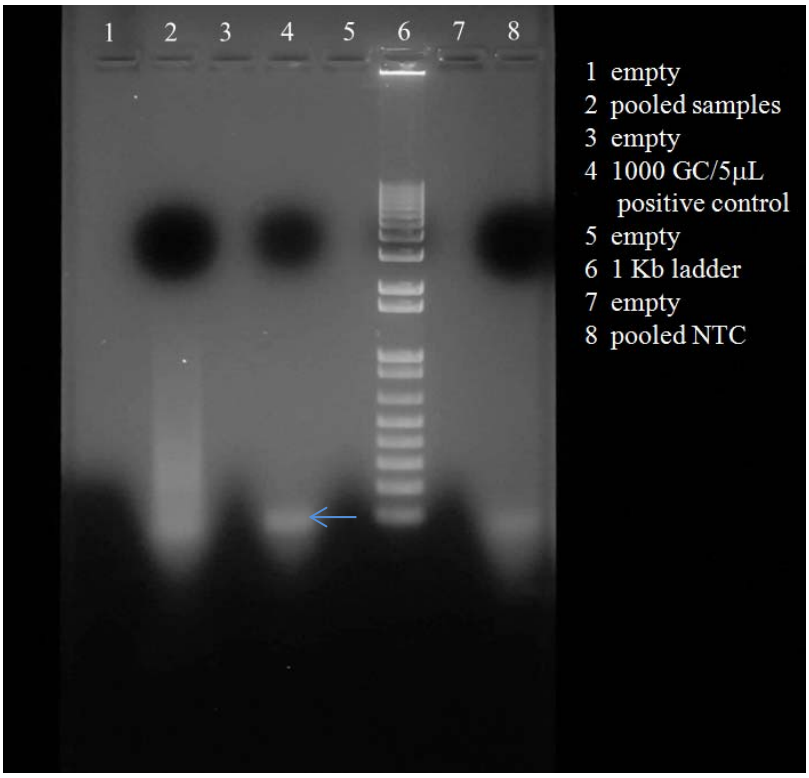

Figure 1. Gel Electrophoresis of AIR-10-21-11 Samples Analyzed by PCR on the ABI 9700 Thermocycler (blue arrow denotes an amplicon at ~82 bp in the positive control well, consistent with *B. atrophaeus rtp*. Bands are visible in the NTC and pooled sample wells, but are migrating slightly lower than the band in well 4, and may be primers).

4.2 Detection of PLGA Microspheres

PLGA microspheres were observed in all positive control samples but at low quantities (i.e. less than 20 microspheres per view). Sample autofluorescence prevented visualization of PLGA in most samples; only samples IRP-WIPE-10-24-11-ABC-0089 and IRP-WIPE-10-24-11-ABC-0099 contained fluorescent particles consistent with the PLGA micropsheres. The gauze and filter matrices are autofluorescent, creating a diffuse green background under the epifluorescent conditions; moreover, irregularly-shaped, autofluorescent particulate matter in and on some sample matrices made it impossible to discern if PLGA microspheres were present. Table 5 lists each sample and the corresponding microscopic descriptions. Representative photos are shown in Figure 2. Several of the samples had begun to support mold growth at the time of microscopy, which also contributes to autofluorescence.

Table 5. Microscopic Observations of PLGA Microspheres

Sample ID	Microscopic Observations

Sample ID	Microscopic Observations
IRP-AIR-10-19-11-ABC-B1	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-19-11-ABC-B2	Diffuse green fluorescence, no PLGA microspheres
IRP-AIR-10-19-11-ABC-B3	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-19-11-ABC-B4	Diffuse green fluorescence, no PLGA microspheres
IRP-AIR-10-19-11-ABC-B5	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-19-11-ABC-B6	Diffuse green fluorescence, no PLGA microspheres
IRP-AIR-10-19-11-ABC-B7	Diffuse green fluorescence, no PLGA microspheres
IRP-AIR-10-19-11-ABC-B8	Diffuse green fluorescence, no PLGA microspheres
IRP-WIPE-10-19-11-ABC-B1	2 fluorescent particles observed, too large to be PLGA microspheres
IRP-WIPE-10-19-11-ABC-B2	1-2 fluorescent particles observed, too large to be PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-19-11-ABC-B3	No PLGA microspheres observed, background fluorescence
IRP-WIPE-10-19-11-ABC-B4	No PLGA microspheres observed, background fluorescence
IRP-WIPE-10-19-11-ABC-B5	No PLGA microspheres observed
IRP-AIR-10-20-11-ABC-001	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-002	Dark field with diffuse green fluorescence; no

Sample ID	Microscopic Observations
	PLGA microspheres
IRP-AIR-10-20-11-ABC-003	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-004	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-005	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-006	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-007	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-20-11-ABC-008	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-009	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-010	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-011	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-012	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-013	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-AIR-10-21-11-ABC-014	Diffuse green background with no fluorescent particles
IRP-AIR-10-21-11-ABC-015	Diffuse green background with no fluorescent particles

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-AIR-10-21-11-ABC-016	Diffuse green background with no fluorescent particles
IRP-AIR-10-21-11-ABC-017	Diffuse green background with no fluorescent particles
IRP-IW-10-20-11-ABC-001	Observed crystalline-like fluorescent shards and spherical fluorescent particles; none discernible as PLGA microspheres
IRP-WIPE-10-20-11-ABC-001	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-20-11-ABC-002	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-20-11-ABC-003	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-20-11-ABC-004	Dark field with diffuse some fluorescence; no PLGA microspheres
IRP-WIPE-10-20-11-ABC-005	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-20-11-ABC-006	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-20-11-ABC-007	Diffuse green background with some fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-20-11-ABC-008	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-20-11-ABC-009	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-20-11-ABC-0010	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0011	Dark field with diffuse green fluorescence; no PLGA microspheres

Sample ID	Microscopic Observations
IRP-WIPE-10-21-11-ABC-0012	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0013	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-21-11-ABC-0014	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0015	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0016	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0017	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0018	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0019	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0020	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0021	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0022	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0023	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0024	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0025	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0026	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0027	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0028	Dark field with diffuse some fluorescence; no PLGA microspheres

Sample ID	Microscopic Observations
IRP-WIPE-10-21-11-ABC-0029	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-21-11-ABC-0030	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0031	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-21-11-ABC-0032	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0033	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0034	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0035	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0036	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0037	Very bright green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0038	Very bright green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0039	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible

Sample ID	Microscopic Observations
	from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0040	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-21-11-ABC-0041	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0042	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0043	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0044	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0045	Diffuse green background with no fluorescent particles

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-21-11-ABC-0046	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0047	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0048	Diffuse green background with no fluorescent particles
IRP-WIPE-10-21-11-ABC-0049	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-050	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-051	Diffuse green background with no fluorescent particles

Sample ID	Microscopic Observations
IRP-WIPE-10-24-11-ABC-052	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-053	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-054	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-055	Light field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-056	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-057	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-058	Dark field with diffuse green fluorescence; no PLGA microspheres
IRP-WIPE-10-24-11-ABC-059	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-060	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-061	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-062	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-063	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-24-11-ABC-064	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible

Sample ID	Microscopic Observations
	from PLGA microspheres
IRP-WIPE-10-24-11-ABC-065	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-066	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-067	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-068	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-069	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-070	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-071	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-072	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-073	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-074	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-075	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-076	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Sample ID	Microscopic Observations
IRP-WIPE-10-24-11-ABC-077	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-078	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-24-11-ABC-079	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-080	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-081	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-082	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-083	Bright green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-084	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-085	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-086	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-087	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-088	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible

Sample ID	Microscopic Observations
	from PLGA microspheres
IRP-WIPE-10-24-11-ABC-089	Dark background with diffuse green fluorescence, 1 fluorescent particle observed consistent with PLGA microsphere
IRP-WIPE-10-24-11-ABC-090	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-091	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-092	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-093	Diffuse green background with many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-094	Dark background with green fluorescence, many fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
IRP-WIPE-10-24-11-ABC-095	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-096	Diffuse green background with few fluorescent particles of irregular size and shape, indiscernible from PLGA microspheres
IRP-WIPE-10-24-11-ABC-097	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-098	Diffuse green background with no fluorescent particles
IRP-WIPE-10-24-11-ABC-099	Very diffuse green background, ~5 fluorescent particles observed consistent with PLGA microspheres

Sample ID	Microscopic Observations
IRP-FPG-10-24-11-ABC-001	N/A
IRP-FPC-10-24-11-ABC-001	N/A
IRP-AIR-10-24-11-ABC-018	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-019	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-020	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-021	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-022	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-023	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-024	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-025	Diffuse green background with no fluorescent particles
IRP-AIR-10-24-11-ABC-027	Diffuse green background with no fluorescent particles
Filter Blank 1	Diffuse green background with no fluorescent particles
Filter Blank 2	Diffuse green background with no fluorescent particles

Table 5. Microscopic Observations of PLGA Microspheres (Continued)

Sample ID	Microscopic Observations
Gauze Blank 1	Diffuse green background with no fluorescent particles
Gauze Blank 2	Green fluorescent background, no PLGA microspheres or fluorescent particles

Sample ID	Microscopic Observations
Gauze Blank 3	Very diffuse green background, no PLGA microspheres or fluorescent particles
Gauze Blank 4	Diffuse green background, no PLGA microspheres or fluorescent particles
Gauze Blank 5	Diffuse green background with no fluorescent particles
Water Blank 1	No fluorescent particles
Grease Blank 1	N/A
Filter PC 1	Many fluorescent PLGA microspheres observed in both membrane and batting layer
Filter PC 2	Very bright green background with ~20 PLGA microspheres visible on the membrane; no PLGA microspheres visible on the batting
Gauze PC 1	Some fluorescent PLGA microspheres observed; fewer than on Filter PC1
Gauze PC 2	Some fluorescent PLGA microspheres observed in background of green autofluorescence
Gauze PC 3	Bright green background with 1 fluorescent particle suspected to be PLGA microsphere
Gauze PC 4	Some fluorescent PLGA microspheres observed (~8) in diffuse green background
Gauze PC 5	Very bright green background, few PLGA microspheres observed
Water PC 1	5 fluorescent PLGA microspheres
Grease PC 1	N/A

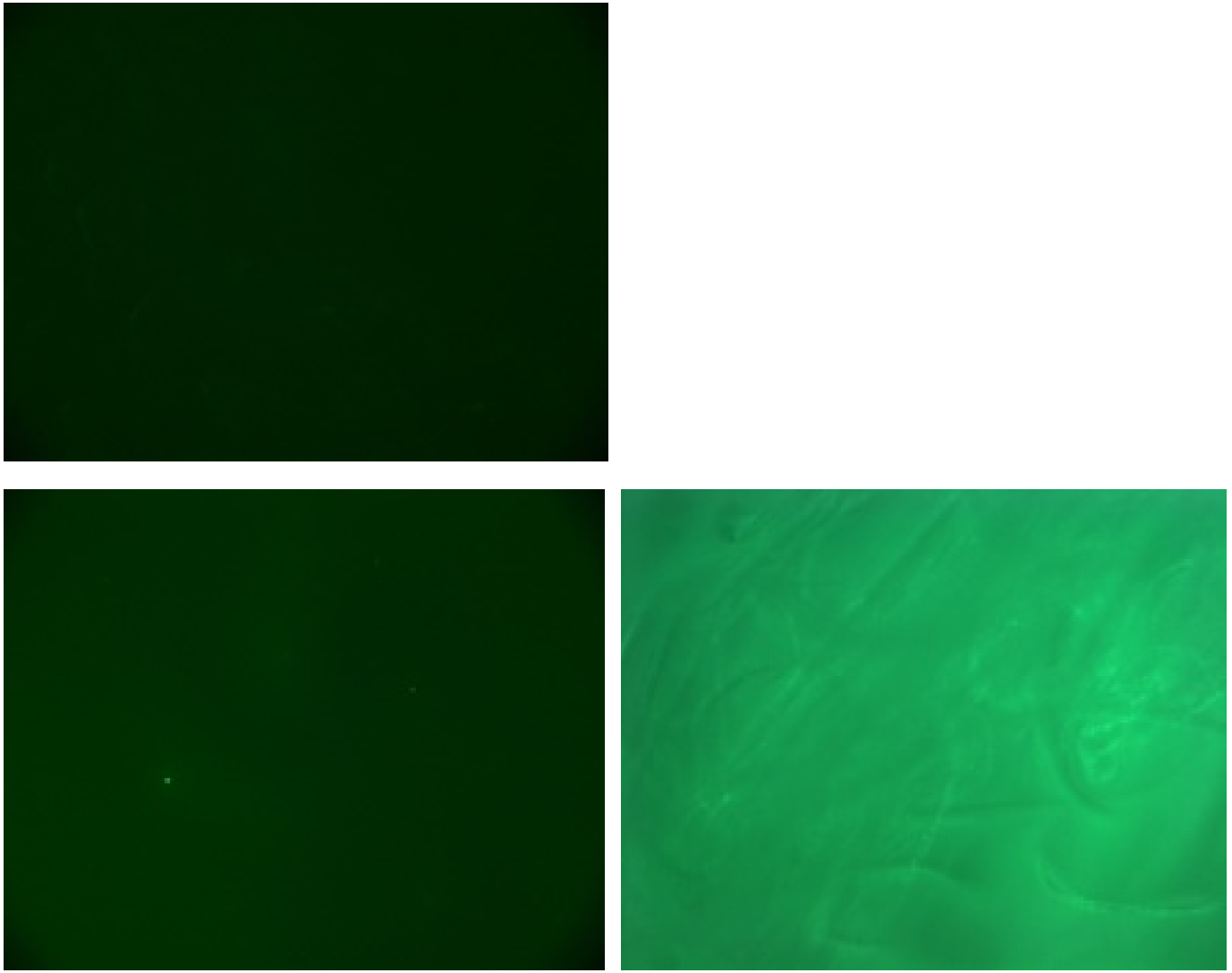

Figure 2. Microscopic Images. Top: IRP-AIR-10-19-11-ABC-B4 (no microspheres present, representative of negative samples), bottom left: Filter PC 1 (two microspheres observed), bottom right: Gauze PC 2 (diffuse green

background, several microspheres present, but not observed as discrete particles).

4.3 Enumeration of Putative Viable *B. atrophaeus* in Archived Samples

None of the filter samples appeared to contain *B. atrophaeus*. Ten gauze samples had putative *B. atrophaeus* colonies (Table 6), including five samples originally reported in the January 18, 2012 Interim Report. Putative *B. atrophaeus* was observed in six of these ten presumptive positives when plated for enumeration, although two samples displayed quantities less than the limit of quantification (<LOQ). The remaining four presumptive positive samples contained no putative *B. atrophaeus* when plated for enumeration; these samples may contain *B. atrophaeus* at levels approaching the limit of detection (LOD). Heat-shock was used to reduce the number of vegetative cells present in the sample extract dilutions. Extract dilutions plated prior to heat-shock (Table 6A) had significantly fewer putative *B. atrophaeus* colonies as compared to extract dilutions plated after heat-shock (Table 6B); thus, heat-shock aided in enumeration of the putative *B. atrophaeus* colonies. The *B. atrophaeus* positive control spikes were recovered at approximately the same concentration before and after heat-shock. *B. atrophaeus* was recovered in the spiked filter positive control, but not from any of the filter test samples, indicating that no *B. atrophaeus* was present on the filters; whether this is because *B. atrophaeus* was not present in the air samples or because the filters did not retain *B. atrophaeus* is not discernible from this data.

4.4 Identification of Background Microbial Flora by Sequence Analysis

4.4.1 Extraction of DNA

Initially, each of the 30 isolates and five pooled samples were extracted following the DNeasy® Gram-positive bacteria protocol (Qiagen, Valencia, CA). The DNeasy® extracts could not be used for PCR due to background 16S rRNA DNA in one of the enzymatic lysis buffer components. A thermolysis technique was used to reduce the number of reagents needed for extraction, thus reducing the likelihood of contamination. This method worked well for five of the 30 isolates, and resulted in a faint PCR product in eight other isolates. These eight faint PCR products were purified and amplified again using the same 8F and 1492R primers.

None of the pooled samples amplified when extracted using the thermolysis technique and Phusion™ polymerase. The pooled samples that were extracted by thermolysis, as well as an aliquot of each pooled sample that had not gone through an extraction method, were cleaned using an OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) and amplified

Table 6. Enumeration of Putative *B. atrophaeus* Colonies in Sample Extracts

A. Prior to Heat-Shock					
Sample Number	Plated Dilution	Average Plate Count[a]			Final Enumeration (CFU/mL)
IRP-WIPE-10-20-11-D-001	1×10^{-1}	0	0	0	0

IRP-WIPE-10-20-11-D-004	1×10^{-1}	0	0	0	0
IRP-WIPE-10-20-11-D-006[b]	1×10^{-1}	<30	36	114	<LOQ[c]
IRP-WIPE-10-20-11-D-007	1×10^{-1}	0	<30	<30	<LOQ
IRP-WIPE-10-20-11-D-008[b]	1×10^{-1}	0	<30	39	<LOQ
IRP-WIPE-10-21-11-D-0012[b]	1×10^{-1}	0	0	0	0
IRP-WIPE-10-21-11-D-0013	1×10^{-1}	0	0	0	0
IRP-WIPE-10-21-11-D-0024[b]	1×10^{-1}	0	0	0	0
IRP-WIPE-10-21-11-D-0029[b,d]	1×10^{-5}	0	0	0	0
IRP-WIPE-10-24-11-D-0059	1×10^{-1}	0	0	0	0
Filter Positive Control	1×10^{-3}	142	142	150	1.45×10^{5}
Gauze Positive Control	1×10^{-3}	38	41	53	4.40×10^{4}
B. After Heat-Shock					
Sample Number	Plated Dilution	Average Plate Count			Final Enumeration (CFU/mL)
IRP-WIPE-10-20-11-D-001	1×10^{-1}	0	0	0	0
IRP-WIPE-10-20-11-D-004	1×10^{-1}	61	<30	47	<LOQ
IRP-WIPE-10-20-11-D-006[b]	1×10^{-1}	159	175	148	1.61×10^{3}
IRP-WIPE-10-20-11-D-007	1×10^{-1}	185	148	186	1.73×10^{3}
IRP-WIPE-10-20-11-D-008[b]	1×10^{-1}	52	60	53	5.5×10^{2}
IRP-WIPE-10-21-11-D-012[b]	1×10^{-1}	53	60	67	6.0×10^{2}
IRP-WIPE-10-21-11-D-013	1×10^{-1}	0	0	0	0
IRP-WIPE-10-21-11-D-0024[b]	1×10^{-1}	<30	0	0	<LOQ
IRP-WIPE-10-21-11-D-0029[b,d]	1×10^{-5}	0	0	0	0
IRP-WIPE-10-24-11-D-0059	1×10^{-1}	0	0	0	0
Filter Positive Control	1×10^{-3}	119	110	113	1.14×10^{5}
Gauze Positive Control	1×10^{-3}	29	32	36	3.23×10^{4}

[a] Individual plate counts <30 are not statistically significant; these counts are reported as <30 and are not included in the calculation for final enumeration.

[b] Originally reported in the January 18, 2012 Interim Report

[c] <LOQ = less than limit of quantitation.

[d]Upon initial plating, this sample contained a lawn of putative *B. atrophaeus*; upon inspection after subsequent dilution and plating, the microorganism did not appear to be *B. atrophaeus*.

using Phire® polymerase. Four of the five pooled samples that had been thermolysed resulted in a PCR product after cleaning, and all five pooled samples that had no prior extraction procedure amplified after removing inhibitors.

4.4.2 Amplification of 16S rRNA

To isolate background microorganisms, 60 samples were plated onto BHIA and incubated overnight at 36 ± 2°C along with a positive control of *B. atrophaeus*. Colonies resembling *B. atrophaeus* positive control were originally reported in 15 of the 60 samples (Table 7). However, after additional review of the data, only one (IRP-IW-10-20-11-ABC-001) actually appeared to contain *B. atrophaeus*, as marked in Table 7. From these 60 samples, 30 colonies with variable morphologies not resembling *B. atrophaeus* were selected for sequence analysis. The 16S rRNA gene was successfully amplified from only 13 of the 30 unknown isolates when using the 8F and 1492R primers. Out of those 13, only five resulted in a clean PCR product with a concentration ≥13 ng/µL. All five of the isolates that resulted in a clean PCR product were able to be identified by sequencing using the ABI 3130.

The majority of the organisms that were isolated either could not be extracted using the thermolysis method, or could not be amplified with the 8F and 1492R primers. While 8F and 1492R primers are considered "universal primers" they are likely not ideal for all bacterial species, and other "universal primers" that target the 16S rRNA gene could potentially be used to amplify a portion of the gene.

Table 7. Samples Containing Colony Morphologies Similar to *B. atrophaeus*

***B. atrophaeus*-Containing Samples**

IRP-IW-10-20-11-ABC-001[1]
IRP-WIPE-10-20-11-ABC002
IRP-WIPE-10-20-11-ABC003
IRP-WIPE-10-20-11-ABC-006
IRP-WIPE-10-20-11-ABC-008
IRP-WIPE-10-21-11-ABC-0011
IRP-WIPE-10-21-11-ABC-0012
IRP-WIPE-10-21-11-ABC-0014
IRP-WIPE-10-21-11-ABC-0017
IRP-WIPE-10-21-11-ABC-0018
IRP-WIPE-10-21-11-ABC-0019
IRP-WIPE-10-21-11-ABC-0024
IRP-WIPE-10-21-11-ABC-0026
IRP-WIPE-10-21-11-ABC-0027
IRP-WIPE-10-21-11-ABC-0029

[1]Upon additional data review, only one sample contained putative *B. atrophaeus*.

4.4.3 Sequencing of 16S rRNA

4.4.3.1 Sequencing of 16S rRNA from Isolated Colonies using Applied Biosystems 3130 Genetic Analyzer. The BLAST nucleotide result with the highest maximum identity percentage is listed in Appendix A, and the top 25 BLAST results, as well as the sequence information obtained, are shown in Appendix B. Six of the 30 unknown isolates resulted in at least one high quality sequencing read. Isolates 4, 19, 22, and 29 are likely *Proteus* species, isolate 15 is likely a *Planomicrobium* species, and isolate 16 is likely a *Curtobacterium* species.

4.4.3.2 Sequencing of 16S rRNA Amplified with Primers 8F and 1492R using Ion Torrent PGM™ Initial sequencing on the PGM™ yielded poor results most likely due to failure of the library preparation. Poor quality 16S DNA amplicons and/or the possibility of carry over inhibitory components may have caused the library preparation to fail. The PGM™ functioned properly and a successful run occurred. After examination of the run, summary evidence pointed to the likelihood that poor clonal amplification had occurred on the OneTouch™. The ISPs loaded correctly into the micron sized wells, and all fluidics and semiconductor functions operated normally; however, template ISPs gave a reading of 8.23% on the Qubit® during the quality analysis check prior to sequencing. The percentage recommended to proceed with sequencing is >50%. Poor clonal amplification was potentially due to poor library construction in the presence of inhibitors that interfered with ligation of the molecular barcodes and adapters; this step is crucial for all other subsequent steps in the library preparation and sequencing. Sequencing reads generated on the PGM™ were of low quality; a quality filtration was performed on the reads using CLCGenomics Workbench software, but there were too few reads post-filtration to perform accurate BLAST analysis or assembly. The reads remaining after filtration were not analogous to anything in the 16S database. Therefore no data was obtained from the PGM™ analysis.

4.4.3.3 Sequencing of 16S rRNA Amplified with Primers 27F and 1492R using Ion Torrent PGM™. The 16S rRNA PCR strategy used was successful in producing amplicons from all five pooled samples. Pools 2-5 gave high quality sequence data resulting from IonTorrent sequencing. Pool 1 did not

yield sufficient high quality data, which is either due to the 16S amplicon quality (source sample influence) or sequencing library and sequencer quality (sequencing influence). Resequencing of pool 1 was not performed due to time and budget constraints.

Table 8 shows the dominant genera of bacteria identified by BLAST search and the most probable species identified by the Battelle QUEST™ method. Figures 2-5 present hierarchically organized relative abundance data resulting from Ion Torrent PGM™ sequence analysis using the KRONA tool. KRONA is an opensource software built with HTML5 (web-browser format) that may ingest BLAST data and prepare visual results of the relative abundances of the total top BLAST hits. The KRONA maps in Figures 2-5 show resolution at the genus level (outer ring of the circle) organized to lower sub-classifications (inner radii of the circle). Percentage of BLAST reads matching each group of bacteria are included in the figure to assist in interpretation. In general, all pools had *Pseudomonas* as the most prevalent genus, ranging from 31-87 % of the total genetics sequences identified (Table 8). Pool five was the least diverse sample with *Pseudomonas* and *Shewanella* species comprising 95% of the sample. Other genera of bacteria discovered in the pools included *Stenotrophomonas, Xanthomonas, Comomonas, Herbaspirilium, Lactobacillus, Acinetobacter,* and *Yersinia*. The Genus *Bacillus* was not observed in pools 2, 4 and 5 and was at a level near to the limit of detection for the methods used in pooled sample 3 (0.04% of the genetic material identified). Further, most of the species identified from pools 2-5 belonged to the phylum *Proteobacteria,* with low observance (<5%) of the phyla *Firmicutes*, *Bacteroidetes* and *Actinomycetales* (Figures 2-5). In general, the pools had similar profiles of bacteria identified by 16S sequencing, varying mostly by abundance of genera between pools.

Table 8. Results of 16S rRNA Sequencing Based on BLAST and QUEST™ Analysis

Sample	Dominant Genera by BLAST	Dominant Organisms by QUEST™ (top 15 most probable species)
Pool 1	ND*	ND
Pool 2	*Pseudomonas (48%)* *Stenotrophomonas (18%)* *Xanthomonas (5%)* *Yersinia (4%)* *Comamonas (4%)*	*Stenotrophomonas_rhizophila_strain_e-p10* *Pseudomonas_fragi_strain_ATCC_4973* *Acidaminococcus_intestini_strain_ADV_255.99* *Stenotrophomonas_maltophilia_strain_IAM_12423* *Acidaminococcus_fermentans_strain_VR4* *Comamonas_kerstersii_strain_LMG_3475* *Simplicispira_metamorpha_strain_DSM_1837* *Comamonas_aquatica_strain_:_LMG_2370* *Pseudomonas_psychrophila_strain_E-3* *Microvirgula_aerodenitrificans_strain_Sgly2* *Pseudomonas_lundensis_strain_ATCC_49968* *Stenotrophomonas_koreensis_strain_TR6-01* *Dysgonomonas_capnocytophagoides_strain_LMG* *Pseudomonas_agarici_strain_71A* *Brevundimonas_terrae_strain_KSL-145*

Table 8. Results of 16S rRNA Sequencing Based on BLAST and QUEST™ Analysis (Continued)

Sample	Dominant Genera by	Dominant Organisms by QUEST™

	BLAST	(top 15 most probable species)
Pool 3	*Pseudomonas (31%)* *Shewanella (18%)* *Acinetobacter (7%)* *Herbaspirillium (6%)* *Stenotrophomonas (4%)* *Lactobacillus (3%)*	*Shewanella_baltica_strain_63* *Stenotrophomonas_rhizophila_strain_e-p10* *Pseudomonas_fragi_strain_ATCC_4973* *Herbaspirillum_autotrophicum_strain_IAM_14942* *Shewanella_morhuae_strain_U1417* *Morganella_psychrotolerans_strain_U2/3* *Herbaspirillum_rhizosphaerae_strain_UMS-37* *Paucimonas_lemoignei_strain_LMG_2207* *Acinetobacter_ursingii_strain_LUH* *Arcobacter_nitrofigilis_strain_CI* *Dysgonomonas_capnocytophagoides_strain_LMG* *Lactobacillus_curvatus_strain_:DSM_20019* *Shewanella_putrefaciens_strain_LMG_26268* *Myroides_odoratimimus_strain_:_CCUG_39352* *Acinetobacter_haemolyticus_strain_DSM_6962*
Pool 4	*Pseudomonas (34%)* *Stenotrophomonas (42%)* *Xanthomonas (10%)* *Pseudoxanthomonas (3%)*	*Stenotrophomonas_rhizophila_strain_e-p10* *Pseudomonas_fragi_strain_ATCC_4973* *Stenotrophomonas_koreensis_strain_TR6-01* *Stenotrophomonas_maltophilia_strain_IAM_12423* *Pseudomonas_hibiscicola_strain_ATCC_19867* *Pseudomonas_psychrophila_strain_E-3* *Stenotrophomonas_nitritireducens_strain_L2* *Pseudomonas_geniculata_strain_ATCC_19374* *Pseudomonas_mucidolens_strain_IAM12406* *Pseudoxanthomonas_spadix_strain_IMMIB_AFH-5* *Mycoplana_bullata_strain_IAM_13153* *Stenotrophomonas_terrae_strain_:_R-32768* *Pseudomonas_extremorientalis_strain_KMM_3447* *Pseudomonas_abietaniphila_strain_:ATCC_700689* *Pseudomonas_moraviensis_strain_CCM_7280*
Pool 5	*Pseudomonas (87%)* *Shewanella (8%)*	*Pseudomonas_fragi_strain_ATCC_4973* *Pseudomonas_agarici_strain_71A* *Shewanella_putrefaciens_strain_LMG_26268* *Pseudomonas_psychrophila_strain_E-3* *Shewanella_baltica_strain_63* *Pseudomonas_lundensis_strain_ATCC_49968* *Pseudomonas_veronii_strain_CIP_104663* *Pseudomonas_libanensis_strain_CIP_105460* *Stenotrophomonas_rhizophila_strain_e-p10_* *Pseudomonas_palleroniana_strain_CFBP_4389* *Shewanella_hafniensis_strain_P010* *Shewanella_oneidensis_strain_MR-1* *Pseudomonas_mucidolens_strain_IAM12406* *Pseudomonas_caricapapayae_strain_Robbs_ENA-378* *Pseudomonas_taetrolens_strain_I11*

*ND = no data

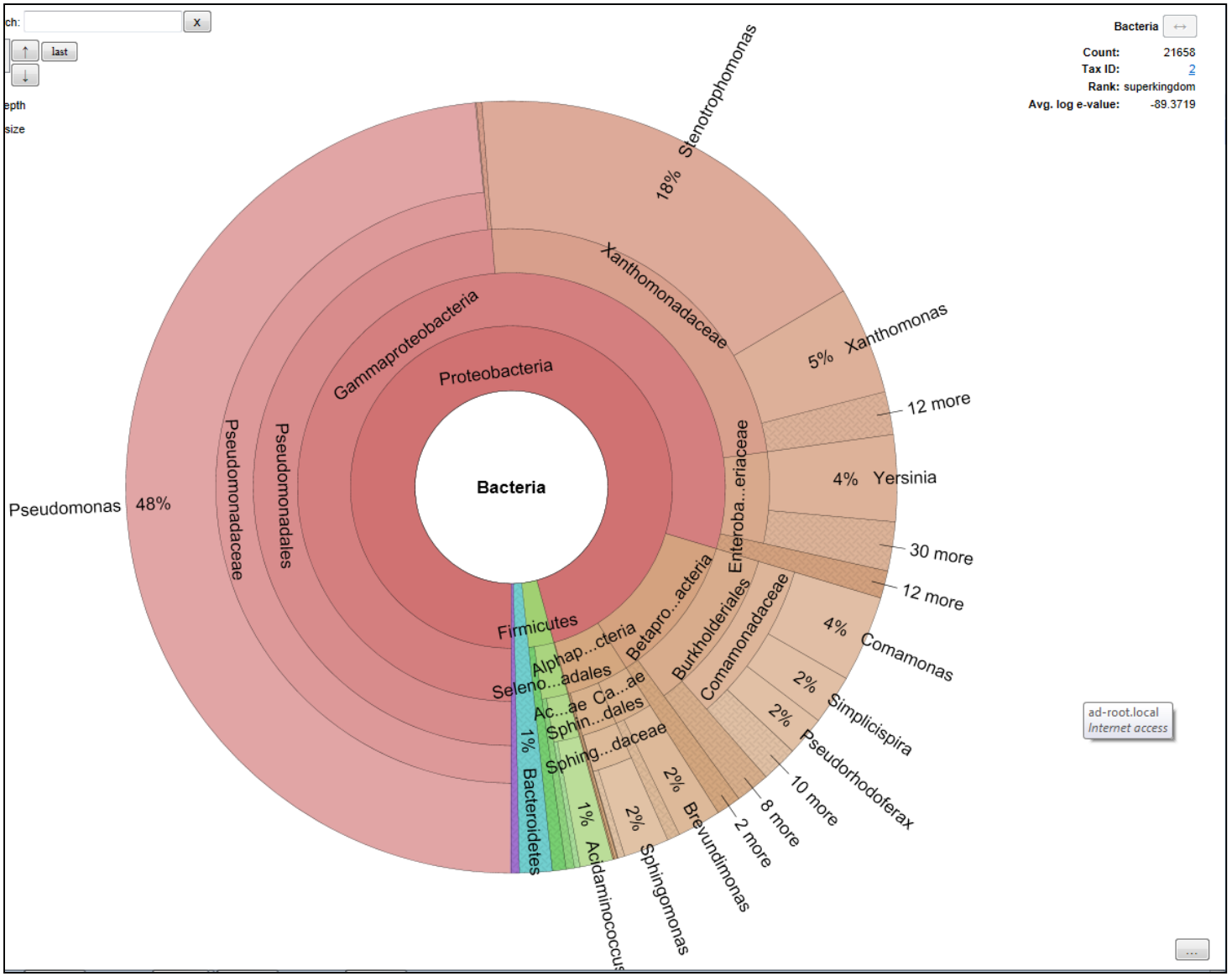

Figure 3. KRONA Visualization of BLAST Results for Pool 2

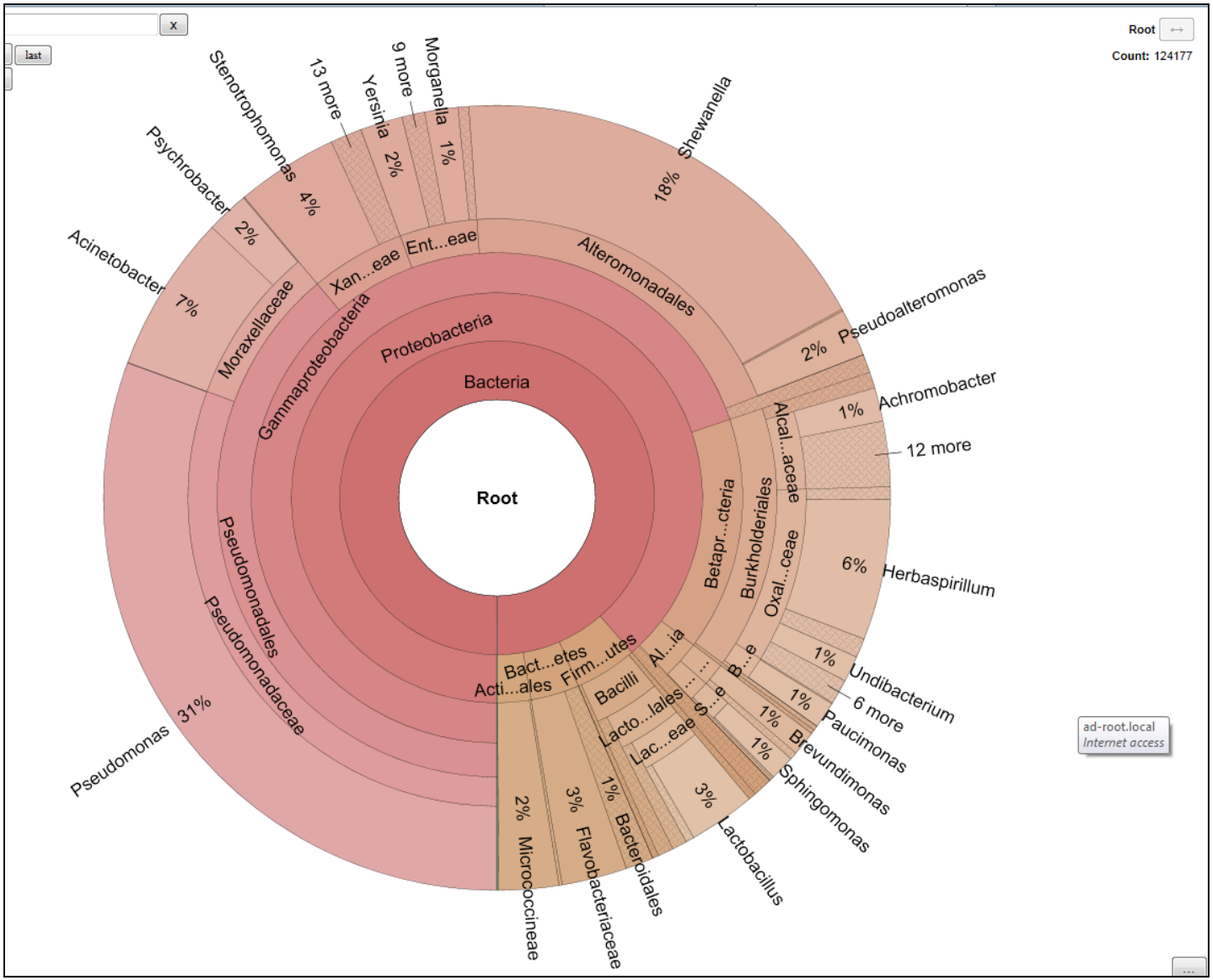

Figure 4. KRONA Visualization of BLAST Results for Pool 3

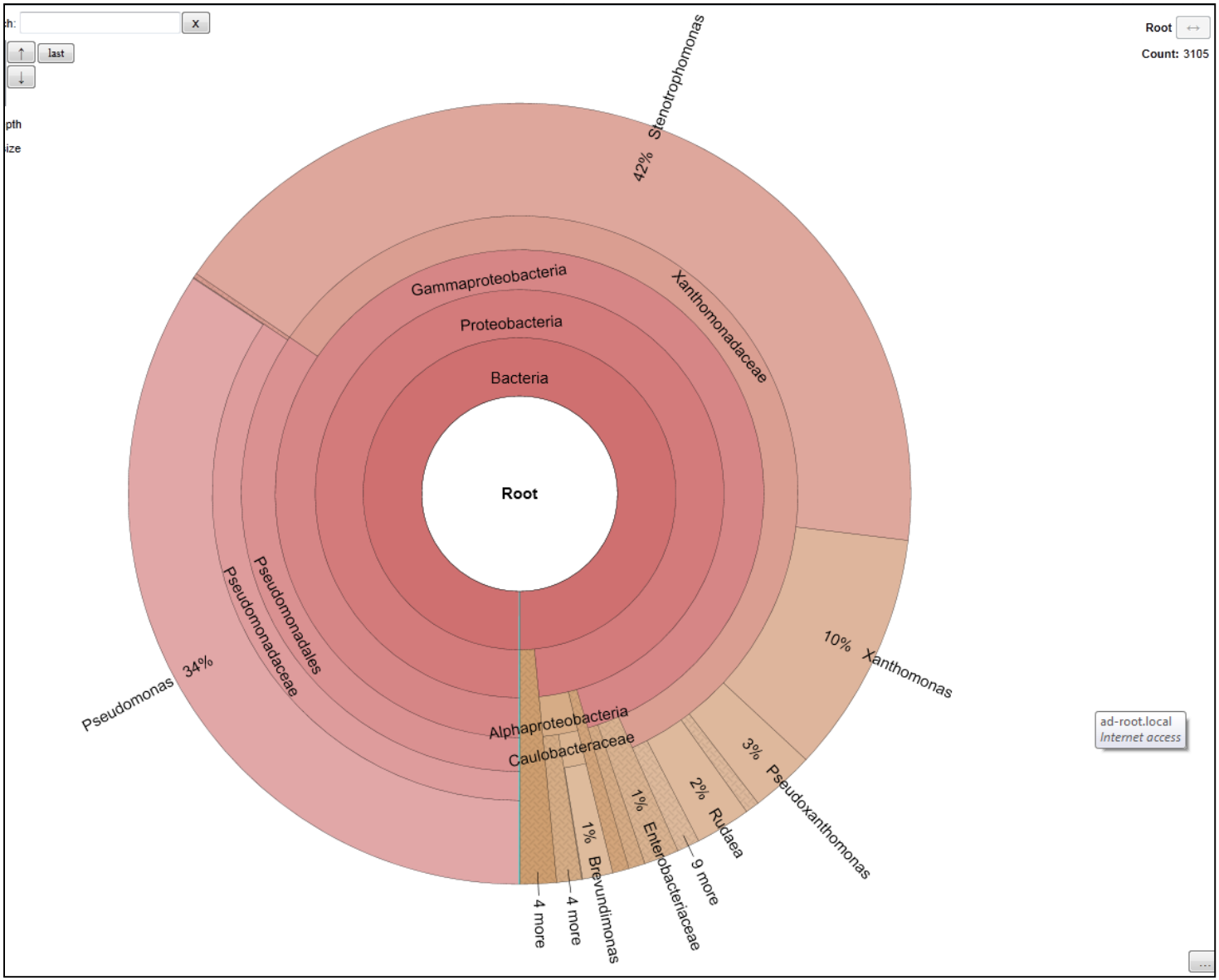

Figure 5. KRONA Visualization of BLAST Results for Pool 4

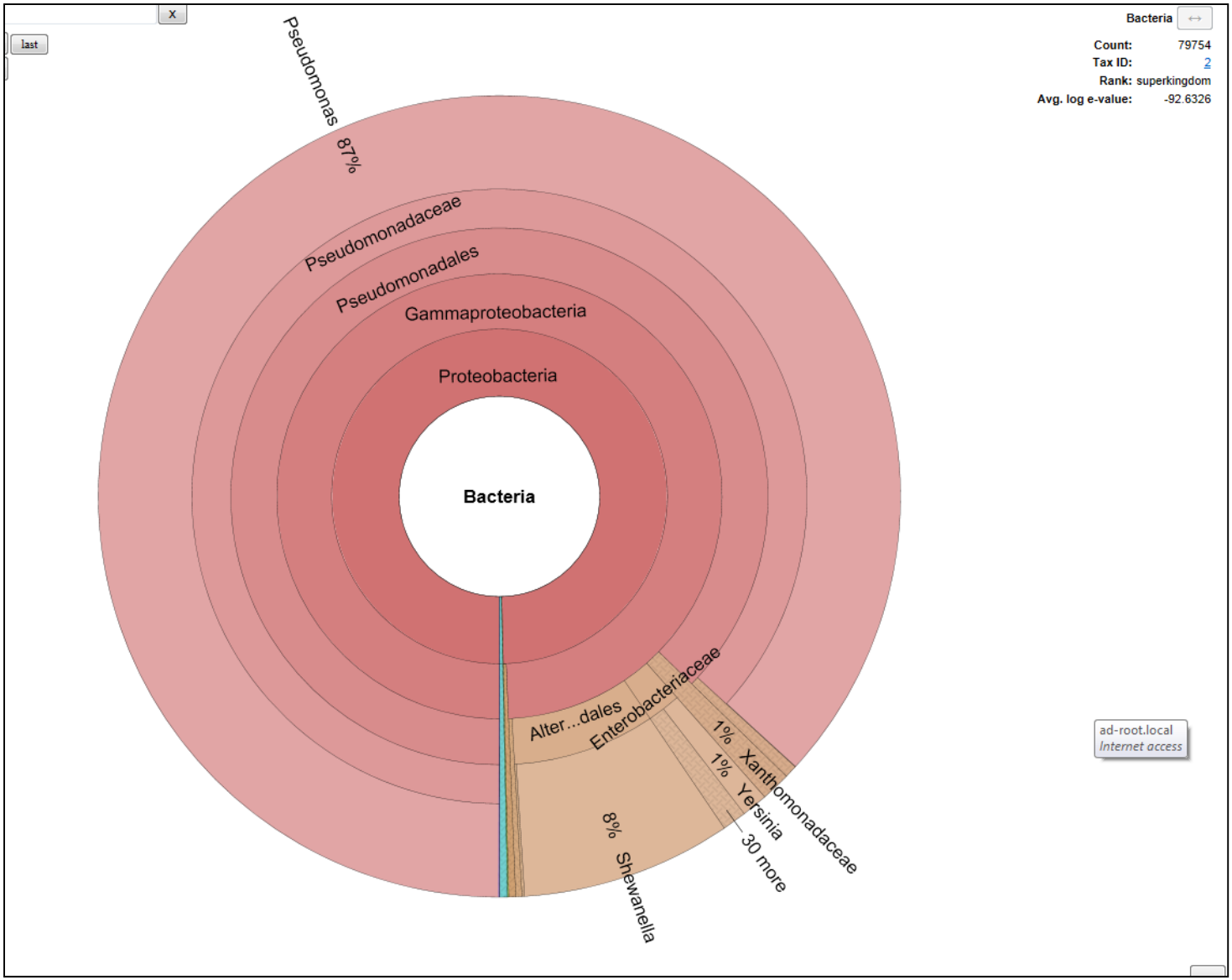

Figure 6. KRONA Visualization of BLAST Results for Pool 5

5.0 SUMMARY

No *B. atrophaeus* DNA was detected in any of the sample extracts. However, viable bacteria very similar to *B. atrophaeus* positive control colony morphology were recovered from 1 of 60 original sample extracts, and from 10 of the archived test samples (five contained putative *B. atrophaeus* in quantities greater than LOQ). Furthermore, *B. atrophaeus* DNA could be extracted from, and detected in, spiked positive controls of pristine gauze and filter matrices prepared from the same lots of gauze and filters as the samples. It is possible that *B. atrophaeus* was present in low quantities and below the limit of detection by qPCR; however, it is more likely that non-detection by qPCR was due to inhibitors present in the sample matrices that carried over during the extraction process, since putative *B. atrophaeus* was recoverable on BHIA.

PLGA microspheres may not be a suitable synthetic surrogate, as they become permanently immobilized in sampling matrices; extraction processes were ineffective at removing PLGA microspheres for quantitation by fluorometer, and autofluorescence from the sample matrices complicated detection of PLGA microspheres via direct microscopic observation.

Amplification of 16S rRNA genes was accomplished in only 13 of 30 attempted reactions from the isolated colonies, and sequence analysis of the 16S rRNA genes was only achieved for six out of these 13 amplicons. The remaining seven amplicons were likely of poor quality and not suitable for sequence analysis. Amplification of 16S rRNA genes is performed using 'universal' primers that are generated to conserved regions in the 16S genes; however, there are several sets of primers that can be used, and PCR conditions may favor certain amplicons over others. If a different primer set is chosen, additional isolates may be identified.

Sequence analysis of the pooled sample extracts was improved using primers 27F and 1492R (as compared to primers 8F and 1492R). *Pseudomonas* was the primary species present in sample pools 2-5. Sequence analysis could not be performed on pool 1; the 16S amplicons were of insufficient quality.

6.0 RECOMMENDATIONs

Many sample extracts were highly inhibitory to qPCR. Inhibition was not attributable to the filter and gauze wipe matrices, as sample extracts from negative and positive controls prepared using identical pristine matrices were either not inhibitive or only slightly inhibitive to qPCR. Inhibition, therefore, must be attributable to sample complexity derived from processing in the rendering facility; potential inhibitors that may have been introduced onto the filter and gauze wipe matrices during sampling include: animal tissues and fluids, particulate matter (e.g., soil, dirt, debris), industrial and mechanical fluids, and other environmental contaminants. Furthermore, *B. atrophaeus* was likely present in trace quantities in many of the samples; putative *B. atrophaeus* was recovered from one of the original sample extracts and 10 of the archived samples, although the isolate identities were not independently confirmed using qPCR or other genotypic or phenotypic assay. A laboratory spiking study in various tissues and in gauze wipes of

tissue handling areas, combined with refined extraction processes would provide limit of detection information and determine suitable extraction methods for mitigating carryover of inhibitory components from the rendering facility.

PLGA microspheres appeared to be irreversibly bound to gauze and filter matrices, preventing efficient extraction of the microspheres into an aqueous solution for detection in the 96-well microtiter plate assay developed for this study. Furthermore, autofluorescence from matrices and other particulate matter in the samples interfered with direct visualization of PLGA microspheres on the sample surfaces. Further testing is warranted to determine if an alternate bead composition would prevent surface interaction and irreversible binding to sample matrices. Alternate fluorophores could be integrated into the beads to improve direct microscopic observations.

From metagenome analysis, *Pseudomonas* was the most prevalent genus present in all of the samples and other genera included, *Stenotrophomonas, Xanthomonas, Comomonas, Herbaspirilium, Lactobacillus, Acinetobacter,* and *Yersinia*. In general, the pools had similar profiles of bacteria identified by 16S sequencing, varying mostly by abundance of genera between pools. In this study, sequencing data from the pure isolates did not correlate with sequencing data from the pooled samples. Because 16S rRNA products were amplified from both pooled extracts and isolated colonies prior to sequencing, it was expected that metagenome sequencing of the pooled sample fractions should contain all of the pure isolate sequences, thus obviating the need to isolate colonies prior to sequencing. However, as previously stated, there are several 'universal' primer sets that can be used to amplify 16S genes and PCR conditions may favor amplification from some targets over others. Primer set 8F and 1492R did not provide clean amplification products in the pooled samples, but it is possible the switch from primer 8F (isolated colonies) to primer 27F (pooled samples) was sufficient to select for an entirely different set of amplicons in the pooled samples. It is also possible that the isolated colonies may represent a very small portion of the entire metagenome population, and while we could cultivate and sequence these organisms, their sequence represents such a small portion of the entire metagenome that it is occluded by the other organisms comprising the population majority.

If further identification of pure isolates is desired, there are several new library kits on the market which could be evaluated for future use; New England Biolabs (Ipswich, MA) has released a new kit specifically made for the PGM™ that can be used for single isolate chip runs and multiplexing runs, and Life Technologies recently released a new library preparation kit that explicitly for bacterial amplicons from environmental samples used for multiplexing. Future work could also include the development of Paired-End sequencing (PES) on the PGM™ to obtain double coverage and crossover sequencing for more robust sequence analysis.

Whole metagenome sequencing without prior amplification of 16S rRNA would definitely increase the amount of information returned, as this is not biased by the amplification of 16S genes and will provide identification of prokaryotic and eukaryotic communities. This process requires 0.5 to 1 μg of DNA, and would be amenable to gauze and filter matrices. However, if the samples were heavily burdened with animal tissues (meat, bone, hair, etc.) it is possible that the results would be biased with mammalian sequences and background prokaryotic and eukaryotic community members may be occluded. Thus, method development and validation using samples spiked with varying amounts of mammalian tissues would be required prior to using whole metagenome sequencing in this setting.

APPENDIX A

SAMPLE LIST, MORPHOLOGY, AND IDENTIFICATION

Isolate number	Sample name	Colony description	PCR result (thermolysis method)	Forward primer sequencing result	Reverse primer sequencing result	BLAST results	Second purification and PCR [yes or no]
1	IRP-AIR-102011-ABC-002	~2 mm beige, transparent, circular, shiny	No amplification	NA	NA	NA	NA
2	IRP-IW-102011-ABC-001-100uL	~2 mm circular, orange, umbonate	No amplification	NA	NA	NA	NA
3	IRP-WIPE-102111-ABC-15-100uL	~2-4 mm colonies, white, shiny, circular	No amplification	NA	NA	NA	NA
4	IRP-WIPE-102111-ABC-0016-100uL	~4 mm colonies, beige, dull, some with spreading irregular edge	Faint band, PCR purified then amplified again.	Poor sequencing quality	~682 quality bases	Proteus	yes
5	IRP-WIPE-102111-ABC-0025-100uL	~4 mm colonies, light beige, umbonate	No amplification	NA	NA	NA	NA
6	IRP-WIPE-102111-ABC-0027-100uL	~4 mm beige, circular	No amplification	NA	NA	NA	NA
7	IRP-AIR-102011-ABC-001	~2 mm, orange, circular, shiny	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	yes
8	IRP-AIR-102111-ABC-0011	~4 mm, translucent-beige, circular	No amplification	NA	NA	NA	NA
9	IRP-AIR-102111-ABC-0012	Pinpoint, beige	No amplification	NA	NA	NA	NA
10	IRP-WIPE-102111-ABC-0013-100uL	Mold-like, slimy, clear edge, with center	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	yes
11	IRP-AIR-102111-ABC-10	~2 mm, circular, orange, shiny	No amplification	NA	NA	NA	NA
12	IRP-AIR-102011-ABC-006	yellow pinpoint, shiny	No amplification	NA	NA	NA	NA
13	IRP-AIR-102011-ABC-006	6-8 mm, beige, circular with irregular edge	No amplification	NA	NA	NA	NA
14	IRP-AIR-102111-ABC-15	~2 mm, white, umbonate, shiny	No amplification	NA	NA	NA	NA

Isolate number	Sample name	Colony description	PCR result (thermolysis method)	Forward primer sequencing result	Reverse primer sequencing result	BLAST results	Second purification and PCR [yes or no]
15	IRP-AIR-102111-ABC-16	2 mm, orange, convex	Amplified	~573 quality bases	~633 quality bases	Planomicrobium	no
16	IRP-AIR-102111-ABC-011	2 mm, convex, beige, shiny	Amplified	~559 okay quality bases	Poor sequencing quality	Curtobacterium	no
17	IRP-WIPE-102111-ABC-0021	4-6 mm, white, circular, convex	No amplification	NA	NA	NA	NA
18	IRP-WIPE-102111-ABC-0024	mold-like, not slimy, dull, irregular spreading edge	No amplification	NA	NA	NA	NA
19	IRP-WIPE-102011-ABC-002-100uL	Swarm-like, smooth lawn	Amplified	~642 quality bases	~675 quality bases	Proteus	no
20	IRP-WIPE-102111-ABC-0029-100uL	4 mm colonies, circular, beige, shiny	No amplification	NA	NA	NA	NA
21	IRP-WIPE-102111-ABC-0026-100uL	3-4 mm colonies, light yellow, convex	No amplification	NA	NA	NA	NA
22	IRP-WIPE-102011-ABC-001-100uL	Smooth lawn, beige	Amplified	~617 quality bases	~675 quality bases	Proteus	no
23	IRP-AIR-102011-ABC-006	1 mm colonies, light orange.	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	NA
24	IRP-AIR-102111-ABC-013	1 mm colonies, yellow, convex	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	NA
25	IRP-WIPE-101911-ABC-B5	Pinpoint pink colonies	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	NA
26	IRP-WIPE-101911-ABC-B2	Branch-like growth, light spreading edge, white, dull	No amplification	NA	NA	NA	NA
27	IRP-WIPE-102011-ABC-004	Lawn, thin, smooth, beige	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	NA

Isolate number	Sample name	Colony description	PCR result (thermolysis method)	Forward primer sequencing result	Reverse primer sequencing result	BLAST results	Second purification and PCR [yes or no]
28	IRP-WIPE-102111-ABC-0011-100uL	lawn, light brown, rippled surface, shiny	No amplification	NA	NA	NA	NA
29	IRP-WIPE-102111-ABC-0012-100 uL	lawn spread throughout, smaller beige colonies where streak lines are, mixed.	Amplified	~244 quality bases	~679 quality	Proteus	no
30	IRP-WIPE-102011-ABC-006-100 uL	lawn spread throughout, smaller beige colonies where streak lines are, mixed.	No amplification	NA	NA	NA	NA
B. atrophaeus, ATCC 9372	Positive control	Beige-orange, ~2mm circular colonies.	Faint band, PCR purified then amplified again.	Poor sequencing quality	Poor sequencing quality	NA	NA

APPENDIX B

BLAST RESULTS

Isolate 4, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1210	1210	100%	0.0	98%
2	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1210	1210	100%	0.0	98%
3	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1199	1199	100%	0.0	98%
4	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1190	1190	100%	0.0	98%
5	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	1164	1164	100%	0.0	97%
6	NR_043646.1	Xenorhabdus kozodoii strain SaV 16S ribosomal RNA, partial sequence	1153	1153	100%	0.0	97%
7	NR_043637.1	Xenorhabdus koppenhoeferi strain USNJ01 16S ribosomal RNA, partial sequence	1149	1149	100%	0.0	97%
8	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	1144	1144	100%	0.0	97%
9	NR_042326.1	Xenorhabdus budapestensis strain :DSM 16342 16S ribosomal RNA, partial sequence	1138	1138	100%	0.0	96%
10	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	1127	1127	100%	0.0	96%
11	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	96%
12	NR_037074.1	Photorhabdus luminescens subsp. luminescens strain Hb 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	96%
13	NR_027194.1	Xenorhabdus japonica strain SK-1T 16S ribosomal RNA, partial sequence	1120	1120	100%	0.0	96%
14	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	96%
15	NR_042820.1	Xenorhabdus bovienii strain DSM4766 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	96%
16	NR_043642.1	Xenorhabdus doucetiae strain FRM16 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
17	NR_026538.1	Dickeya paradisiaca strain LMG 2542 16S ribosomal RNA, partial sequence	1112	1112	100%	0.0	96%

Isolate 4, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
18	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%
19	NR_042062.1	Serratia liquefaciens strain CIP 103238 16S ribosomal RNA, partial sequence	1105	1105	100%	0.0	96%
20	NR_025340.1	Serratia grimesii strain DSM 30063 16S ribosomal RNA, partial sequence	1105	1105	100%	0.0	96%
21	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	1099	1099	100%	0.0	95%
22	NR_025334.1	Obesumbacterium proteus strain 42 16S ribosomal RNA, partial sequence	1099	1099	100%	0.0	95%
23	NR_036851.1	Photorhabdus asymbiotica subsp. asymbiotica strain 3265-8 16S ribosomal RNA, partial sequence	1099	1099	100%	0.0	95%
24	NR_025316.1	Pectobacterium carotovorum subsp. odoriferum strain LMG 17566 16S ribosomal RNA, partial sequence	1098	1098	100%	0.0	95%
25	NR_029011.1	Photorhabdus luminescens subsp. kayaii strain 1121 16S ribosomal RNA, complete sequence	1096	1096	100%	0.0	95%

Isolate 4, Reverse Primer, sequence

CGATTCCGACTTCATGGAGTCGAGTTGCANACTCCAATCCGGANTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGNCNCTTCTCTTTGTATCTGNCATTGTAGC
ACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCNCCATTACGCGCTGGCAAC
AAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACTCCTCTATCT
CTAAAGGATTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAAC
CTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGT
ATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACA
CGTGGAATTCTACCCCCCTCT

Isolate 15, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_044384.1	Planomicrobium glaciei strain 0423 16S ribosomal RNA, partial sequence	1044	1044	100%	0.0	99%
2	NR_025864.1	Planomicrobium okeanokoites strain IFO 12536 16S ribosomal RNA, partial sequence	990	990	100%	0.0	98%
3	NR_025011.1	Planomicrobium koreense strain JG07 16S ribosomal RNA, partial sequence	983	983	100%	0.0	98%
4	NR_025553.1	Planococcus rifietoensis strain M8 16S ribosomal RNA, partial sequence	983	983	100%	0.0	98%
5	NR_025592.1	Planococcus maitriensis strain S1 16S ribosomal RNA, partial sequence	977	977	100%	0.0	97%
6	NR_024881.1	Planomicrobium mcmeekinii strain S23F2 16S ribosomal RNA, partial sequence	974	974	100%	0.0	97%
7	NR_044073.1	Planococcus donghaensis strain JH1 16S ribosomal RNA, partial sequence	972	972	100%	0.0	97%
8	NR_025247.1	Planococcus maritimus strain TF-9 16S ribosomal RNA, partial sequence	972	972	100%	0.0	97%
9	NR_042259.1	Planomicrobium chinense strain : DX3-12 16S ribosomal RNA, partial sequence	970	970	100%	0.0	97%
10	NR_028950.1	Planomicrobium psychrophilum strain CMS 53or 16S ribosomal RNA, partial sequence	961	961	100%	0.0	97%
11	NR_025469.1	Planococcus antarcticus strain CMS 26or 16S ribosomal RNA, partial sequence	961	961	100%	0.0	97%
12	NR_026090.1	Planococcus citreus strain NCIMB 1493 16S ribosomal RNA, partial sequence	957	957	100%	0.0	97%
13	NR_025781.1	Planococcus stackebrandtii strain K22-03 16S ribosomal RNA, partial sequence	948	948	100%	0.0	97%
14	NR_024864.1	Planomicrobium alkanoclasticum strain MAE2 16S ribosomal RNA, partial sequence	935	935	100%	0.0	96%

Isolate 15, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
15	NR_026091.1	Planococcus kocurii strain NCIMB 629 16S ribosomal RNA, partial sequence	935	935	100%	0.0	96%
16	NR_043267.1	Bacillus infantis strain SMC 4352-1 16S ribosomal RNA, partial sequence	933	933	100%	0.0	96%
17	NR_041359.1	Sporosarcina saromensis strain HG645 16S ribosomal RNA, partial sequence	917	917	100%	0.0	95%
18	NR_043527.1	Sporosarcina soli strain I80 16S ribosomal RNA, partial sequence	911	911	100%	0.0	95%
19	NR_043526.1	Sporosarcina koreensis strain F73 16S ribosomal RNA, partial sequence	911	911	100%	0.0	95%
20	NR_043682.1	Bacillus kribbensis strain BT080 16S ribosomal RNA, partial sequence	909	909	100%	0.0	95%
21	NR_042395.1	Planococcus columbae strain : PgEx11 16S ribosomal RNA, partial sequence	907	907	100%	0.0	95%
22	NR_043084.1	Bacillus koreensis strain BR030 16S ribosomal RNA, partial sequence	904	904	99%	0.0	95%
23	NR_043268.1	Bacillus idriensis strain SMC 4352-2 16S ribosomal RNA, partial sequence	900	900	100%	0.0	95%
24	NR_042274.1	Bacillus foraminis strain : CV53 16S ribosomal RNA, complete sequence	900	900	100%	0.0	95%
25	NR_042726.1	Bacillus circulans 16S ribosomal RNA, partial sequence	900	900	100%	0.0	95%

Isolate 15, Forward Primer, sequence

GAAAGACGGTTTCGGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCCACGATGCGTAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
AAGGTTTTCGGATCGTAAAACTCTGTTGCGAGGGAAGAAACCGTGCCAAGTAACTANTGGCACCTTGACGGTACCTCGCCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTTTCT
GGTCTGTAACTGACGCTGAGGCGCGAAAGCGTG

Isolate 15, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_044384.1	Planomicrobium glaciei strain 0423 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	98%
2	NR_024881.1	Planomicrobium mcmeekinii strain S23F2 16S ribosomal RNA, partial sequence	1094	1094	100%	0.0	98%
3	NR_025011.1	Planomicrobium koreense strain JG07 16S ribosomal RNA, partial sequence	1092	1092	99%	0.0	98%
4	NR_042259.1	Planomicrobium chinense strain : DX3-12 16S ribosomal RNA, partial sequence	1088	1088	100%	0.0	98%
5	NR_028950.1	Planomicrobium psychrophilum strain CMS 53or 16S ribosomal RNA, partial sequence	1086	1086	99%	0.0	98%
6	NR_025864.1	Planomicrobium okeanokoites strain IFO 12536 16S ribosomal RNA, partial sequence	1081	1081	100%	0.0	97%
7	NR_025553.1	Planococcus rifietoensis strain M8 16S ribosomal RNA, partial sequence	1077	1077	100%	0.0	97%
8	NR_025247.1	Planococcus maritimus strain TF-9 16S ribosomal RNA, partial sequence	1072	1072	100%	0.0	97%
9	NR_024864.1	Planomicrobium alkanoclasticum strain MAE2 16S ribosomal RNA, partial sequence	1066	1066	99%	0.0	97%
10	NR_042395.1	Planococcus columbae strain : PgEx11 16S ribosomal RNA, partial sequence	1062	1062	100%	0.0	97%
11	NR_025592.1	Planococcus maitriensis strain S1 16S ribosomal RNA, partial sequence	1059	1059	96%	0.0	98%
12	NR_025781.1	Planococcus stackebrandtii strain K22-03 16S ribosomal RNA, partial sequence	1042	1042	99%	0.0	96%
13	NR_043526.1	Sporosarcina koreensis strain F73 16S ribosomal RNA, partial sequence	1038	1038	100%	0.0	96%

Isolate 15, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
14	NR_025469.1	Planococcus antarcticus strain CMS 26or 16S ribosomal RNA, partial sequence	1037	1037	100%	0.0	96%
15	NR_044073.1	Planococcus donghaensis strain JH1 16S ribosomal RNA, partial sequence	1035	1035	100%	0.0	96%
16	NR_043527.1	Sporosarcina soli strain I80 16S ribosomal RNA, partial sequence	1033	1033	100%	0.0	96%
17	NR_026090.1	Planococcus citreus strain NCIMB 1493 16S ribosomal RNA, partial sequence	1033	1033	96%	0.0	97%
18	NR_025049.1	Sporosarcina aquimarina strain SW28 16S ribosomal RNA, partial sequence	1027	1027	100%	0.0	96%
19	NR_043720.1	Paenisporosarcina quisquiliarum strain SK 55 16S ribosomal RNA, partial sequence	1026	1026	99%	0.0	96%
20	NR_044122.1	Sporosarcina sp. N-05 strain N-05 16S ribosomal RNA, partial sequence	1022	1022	100%	0.0	96%
21	NR_025573.1	Sporosarcina macmurdoensis strain CMS 21w 16S ribosomal RNA, partial sequence	1016	1016	99%	0.0	96%
22	NR_041359.1	Sporosarcina saromensis strain HG645 16S ribosomal RNA, partial sequence	1011	1011	100%	0.0	95%
23	NR_044193.1	Bacillus ginsengi strain ge14 16S ribosomal RNA, partial sequence	1011	1011	100%	0.0	95%
24	NR_025409.1	Bacillus psychrodurans strain DSM 11713 16S ribosomal RNA, partial sequence	1011	1011	100%	0.0	95%
25	NR_025408.1	Bacillus psychrotolerans strain DSM 11706 16S ribosomal RNA, partial sequence	1011	1011	100%	0.0	95%

Isolate 15, Reverse Primer, sequence

GGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATGCTGAGCCAKGATCAAACTCT
NGANCCGGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAACGGTTTTCTGGGATTGGCTCCCCCTCGCGGGTTGGCAACCCTTTGTACCGTCCATTGTAG
CACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACT
AAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCGCTGTCCCCGAAGGGAAAGCCTTGTC
TCCAAGGCGGTCAGCGGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTC
AGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTC

Isolate 16, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_026156.1	Curtobacterium citreum strain DSM 20528 16S ribosomal RNA, partial sequence	913	913	100%	0.0	94%
2	NR_026157.1	Curtobacterium luteum strain DSM 20542 16S ribosomal RNA, partial sequence	911	911	100%	0.0	94%
3	NR_025467.1	Curtobacterium flaccumfaciens pv. flaccumfaciens strain LMG 3645 16S ribosomal RNA, partial sequence	907	907	100%	0.0	94%
4	NR_041495.1	Curtobacterium ammoniigenes strain NBRC 101786 16S ribosomal RNA, partial sequence	907	907	100%	0.0	94%
5	NR_042315.1	Curtobacterium pusillum strain : DSM 20527 16S ribosomal RNA, complete sequence	907	907	100%	0.0	94%
6	NR_036885.1	Curtobacterium albidum strain IFO 15078 16S ribosomal RNA, partial sequence	887	887	98%	0.0	94%
7	NR_044240.1	Leifsonia kribbensis strain MSL-13 16S ribosomal RNA, partial sequence	874	874	100%	0.0	93%
8	NR_043663.1	Leifsonia shinshuensis strain DB102; JCM10591 16S ribosomal RNA, partial sequence	857	857	100%	0.0	92%
9	NR_043662.1	Leifsonia naganoensis strain DB103; JCM10592 16S ribosomal RNA, partial sequence	857	857	100%	0.0	92%
10	NR_041812.1	Corynebacterium bovis strain ATCC13722 16S ribosomal RNA, partial sequence	857	857	100%	0.0	93%
11	NR_029264.1	Pseudoclavibacter helvolus strain DSM 20419 16S ribosomal RNA, partial sequence	857	857	100%	0.0	93%

Isolate 16, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
12	NR_027523.1	Leifsonia xyli subsp. cynodontis JCM 9733 16S ribosomal RNA, partial sequence	857	857	100%	0.0	92%
13	NR_028739.1	Leifsonia poae strain VKM Ac-1401 16S ribosomal RNA, partial sequence	852	852	100%	0.0	92%
14	NR_025461.1	Curtobacterium herbarum strain P 420/07 16S ribosomal RNA, partial sequence	848	848	100%	0.0	92%
15	NR_043412.1	Leifsonia aquatica strain JCM 1368 16S ribosomal RNA, complete sequence	848	848	99%	0.0	92%
16	NR_043982.1	Leucobacter chromiireducens subsp. solipictus strain TAN 31504 16S ribosomal RNA, partial sequence	846	846	100%	0.0	92%
17	NR_041045.1	Plantibacter auratus strain IAM 18417 16S ribosomal RNA, partial sequence	846	846	100%	0.0	92%
18	NR_025976.1	Clavibacter michiganensis subspecies tessellarius strain 78181 16S ribosomal RNA, partial sequence	846	846	100%	0.0	92%
19	NR_036892.1	Clavibacter michiganensis strain DSM 46364 16S ribosomal RNA, partial sequence	846	846	100%	0.0	92%
20	NR_042669.1	Leifsonia kafniensis strain : KFC-22 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%
21	NR_042287.1	Leucobacter chromiireducens subsp. chromiireducens strain : L-1 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%
22	NR_036947.1	Clavibacter michiganensis subsp. insidiosus strain Burkholder Pb 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%
23	NR_024679.1	Mycetocola lacteus strain CM-10 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%
24	NR_024678.1	Mycetocola saprophilus strain CM-01 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%
25	NR_024677.1	Mycetocola tolaasinivorans strain CM-05 16S ribosomal RNA, partial sequence	841	841	100%	0.0	92%

Isolate 16, Forward Primer, sequence

ACTGANACACNGCCCANACTCCTACGGGAGGCAGNAGNGGNGAATATTGCNNAATGGGCGAAAGCCTGATGCANCNNCNCCGCGTGAGGNATGACNGCCTTCNGGTTG
TAAACNTNTTTTAGTAGGGAAGAANCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAACTACNTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTGTCCG
GAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAATCCCGAGGCTCAACCTCGGGCTTGCAGTGGGTACGGGCAGACTANAGTGCGGTAGG
GGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGANNAGCGAAAGCGNG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTANACGTTGNGCGCTAGATGTAGGGACCTTTCCACGGTTTCTGTGTNGTANCTNACNCATTAAGCGCCCC
GCCTGNNGAGTACGGCC

Isolate 19, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1170	1170	100%	0.0	99%
2	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	98%
3	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	98%
4	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1079	1079	94%	0.0	99%
5	NR_043751.1	Morganella morganii strain DSM 14850 16S ribosomal RNA, partial sequence	989	989	99%	0.0	95%
6	NR_043750.1	Morganella psychrotolerans strain U2/3 16S ribosomal RNA, partial sequence	983	983	99%	0.0	94%
7	NR_042412.1	Providencia heimbachae strain : DSM 3591 16S ribosomal RNA, complete sequence	974	974	100%	0.0	94%
8	NR_042415.1	Providencia vermicola strain : OP1 16S ribosomal RNA, complete sequence	966	966	99%	0.0	94%

Isolate 19, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
9	NR_024848.1	Providencia stuartii strain ATCC 29914 16S ribosomal RNA, partial sequence	966	966	99%	0.0	94%
10	NR_042413.1	Providencia rettgeri strain : DSM 4542 16S ribosomal RNA, complete sequence	961	961	99%	0.0	94%
11	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	961	961	99%	0.0	94%
12	NR_028938.1	Morganella morganii strain M11 16S ribosomal RNA, partial sequence	959	959	99%	0.0	94%
13	NR_041978.1	Pantoea agglomerans strain DSM 3493 16S ribosomal RNA, partial sequence	937	937	98%	0.0	93%
14	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	933	933	100%	0.0	93%
15	NR_042053.1	Providencia alcalifaciens DSM 30120 strain CIP8290T (ATCC9886T) 16S ribosomal RNA, complete sequence	933	933	99%	0.0	93%
16	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	931	931	100%	0.0	93%
17	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	931	931	99%	0.0	93%
18	NR_043645.1	Xenorhabdus mauleonii strain VC01 16S ribosomal RNA, partial sequence	926	926	99%	0.0	93%
19	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	926	926	100%	0.0	93%
20	NR_042811.1	Arsenophonus nasoniae strain ATCC 49151 16S ribosomal RNA, partial sequence	922	922	100%	0.0	93%
21	NR_043643.1	Xenorhabdus griffiniae strain ID10 16S ribosomal RNA, partial sequence	922	922	100%	0.0	93%
22	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	922	922	100%	0.0	92%
23	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	920	920	99%	0.0	93%
24	NR_024644.1	Serratia rubidaea strain JCM1240 16S ribosomal RNA, partial sequence	917	917	100%	0.0	92%
25	NR_043644.1	Xenorhabdus miraniensis strain Q1 16S ribosomal RNA, partial sequence	915	915	99%	0.0	92%

Isolate 19, Forward Primer, sequence

GCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACGGACC
AAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGA
TGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGA
AGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTRTCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTGGG
AATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCG

Isolate 19, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1242	1242	100%	0.0	99%
2	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1230	1230	100%	0.0	99%
3	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1203	1203	100%	0.0	99%
4	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	1195	1195	100%	0.0	99%
5	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1182	1182	100%	0.0	98%
6	NR_043637.1	Xenorhabdus koppenhoeferi strain USNJ01 16S ribosomal RNA, partial sequence	1175	1175	100%	0.0	98%
7	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	1164	1164	100%	0.0	98%

Isolate 19, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
8	NR_043646.1	Xenorhabdus kozodoii strain SaV 16S ribosomal RNA, partial sequence	1162	1162	100%	0.0	98%
9	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	1158	1158	100%	0.0	98%
10	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	1153	1153	100%	0.0	97%
11	NR_042326.1	Xenorhabdus budapestensis strain :DSM 16342 16S ribosomal RNA, partial sequence	1147	1147	100%	0.0	97%
12	NR_027194.1	Xenorhabdus japonica strain SK-1T 16S ribosomal RNA, partial sequence	1134	1134	100%	0.0	97%
13	NR_043642.1	Xenorhabdus doucetiae strain FRM16 16S ribosomal RNA, partial sequence	1129	1129	100%	0.0	97%
14	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	1129	1129	100%	0.0	97%
15	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	1125	1125	100%	0.0	97%
16	NR_042820.1	Xenorhabdus bovienii strain DSM4766 16S ribosomal RNA, partial sequence	1125	1125	100%	0.0	97%
17	NR_042328.1	Xenorhabdus szentirmaii strain :DSM 16338 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
18	NR_037074.1	Photorhabdus luminescens subsp. luminescens strain Hb 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
19	NR_025334.1	Obesumbacterium proteus strain 42 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
20	NR_042821.1	Xenorhabdus nematophila strain DSM3370 16S ribosomal RNA, partial sequence	1112	1112	100%	0.0	96%
21	NR_026538.1	Dickeya paradisiaca strain LMG 2542 16S ribosomal RNA, partial sequence	1110	1110	100%	0.0	96%
22	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	1109	1109	100%	0.0	96%
23	NR_037112.1	Serratia proteamaculans strain 4364 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%
24	NR_041972.1	Erwinia chrysanthemi strain DSM 4610 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%

Isolate 19, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
25	NR_036851.1	Photorhabdus asymbiotica subsp. asymbiotica strain 3265-8 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%

Isolate 19, Reverse Primer, sequence

TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGTGTAGCCCTACTC
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGATAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCC
CAGGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
CCCACGCTTTCGCAC

Isolate 22, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	99%
2	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1079	1079	100%	0.0	98%
3	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1068	1068	100%	0.0	98%

Isolate 22, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
4	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1024	1024	93%	0.0	99%
5	NR_043751.1	Morganella morganii strain DSM 14850 16S ribosomal RNA, partial sequence	941	941	100%	0.0	94%
6	NR_043750.1	Morganella psychrotolerans strain U2/3 16S ribosomal RNA, partial sequence	941	941	100%	0.0	94%
7	NR_042412.1	Providencia heimbachae strain : DSM 3591 16S ribosomal RNA, complete sequence	931	931	100%	0.0	94%
8	NR_042415.1	Providencia vermicola strain : OP1 16S ribosomal RNA, complete sequence	922	922	100%	0.0	94%
9	NR_024848.1	Providencia stuartii strain ATCC 29914 16S ribosomal RNA, partial sequence	922	922	100%	0.0	94%
10	NR_042413.1	Providencia rettgeri strain : DSM 4542 16S ribosomal RNA, complete sequence	917	917	100%	0.0	94%
11	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	917	917	100%	0.0	94%
12	NR_028938.1	Morganella morganii strain M11 16S ribosomal RNA, partial sequence	911	911	100%	0.0	93%
13	NR_042053.1	Providencia alcalifaciens DSM 30120 strain CIP8290T (ATCC9886T) 16S ribosomal RNA, complete sequence	891	891	100%	0.0	93%
14	NR_041978.1	Pantoea agglomerans strain DSM 3493 16S ribosomal RNA, partial sequence	891	891	100%	0.0	93%
15	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	885	885	100%	0.0	93%
16	NR_043645.1	Xenorhabdus mauleonii strain VC01 16S ribosomal RNA, partial sequence	885	885	100%	0.0	93%
17	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	885	885	99%	0.0	93%
18	NR_042811.1	Arsenophonus nasoniae strain ATCC 49151 16S ribosomal RNA, partial sequence	880	880	100%	0.0	92%
19	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	880	880	100%	0.0	92%
20	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	880	880	100%	0.0	92%

Isolate 22, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
21	NR_024644.1	Serratia rubidaea strain JCM1240 16S ribosomal RNA, partial sequence	880	880	100%	0.0	92%
22	NR_043643.1	Xenorhabdus griffiniae strain ID10 16S ribosomal RNA, partial sequence	874	874	100%	0.0	92%
23	NR_043642.1	Xenorhabdus doucetiae strain FRM16 16S ribosomal RNA, partial sequence	874	874	100%	0.0	92%
24	NR_041974.1	Erwinia mallotivora strain DSM 4565 16S ribosomal RNA, partial sequence	874	874	100%	0.0	92%
25	NR_026045.1	Pantoea ananatis strain 1846 16S ribosomal RNA, partial sequence	872	872	100%	0.0	92%

Isolate 22, Forward Primer, sequence

GCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACG
GACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
ATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTG
CCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAACTTG
GGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATG

Isolate 22, Reverse Primer

#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1242	1242	100%	0.0	99%
2	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1230	1230	100%	0.0	99%
3	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1203	1203	100%	0.0	99%
4	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	1195	1195	100%	0.0	99%
5	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1182	1182	100%	0.0	98%
6	NR_043637.1	Xenorhabdus koppenhoeferi strain USNJ01 16S ribosomal RNA, partial sequence	1175	1175	100%	0.0	98%
7	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	1164	1164	100%	0.0	98%
8	NR_043646.1	Xenorhabdus kozodoii strain SaV 16S ribosomal RNA, partial sequence	1162	1162	100%	0.0	98%
9	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	1158	1158	100%	0.0	98%
10	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	1153	1153	100%	0.0	97%
11	NR_042326.1	Xenorhabdus budapestensis strain :DSM 16342 16S ribosomal RNA, partial sequence	1147	1147	100%	0.0	97%
12	NR_027194.1	Xenorhabdus japonica strain SK-1T 16S ribosomal RNA, partial sequence	1134	1134	100%	0.0	97%
13	NR_043642.1	Xenorhabdus doucetiae strain FRM16 16S ribosomal RNA, partial sequence	1129	1129	100%	0.0	97%
14	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	1129	1129	100%	0.0	97%
15	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	1125	1125	100%	0.0	97%
16	NR_042820.1	Xenorhabdus bovienii strain DSM4766 16S ribosomal RNA, partial sequence	1125	1125	100%	0.0	97%
17	NR_042328.1	Xenorhabdus szentirmaii strain :DSM 16338 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
18	NR_037074.1	Photorhabdus luminescens subsp. luminescens strain Hb 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%

Isolate 22, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
19	NR_025334.1	Obesumbacterium proteus strain 42 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%
20	NR_042821.1	Xenorhabdus nematophila strain DSM3370 16S ribosomal RNA, partial sequence	1112	1112	100%	0.0	96%
21	NR_026538.1	Dickeya paradisiaca strain LMG 2542 16S ribosomal RNA, partial sequence	1110	1110	100%	0.0	96%
22	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	1109	1109	100%	0.0	96%
23	NR_037112.1	Serratia proteamaculans strain 4364 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%
24	NR_041972.1	Erwinia chrysanthemi strain DSM 4610 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%
25	NR_036851.1	Photorhabdus asymbiotica subsp. asymbiotica strain 3265-8 16S ribosomal RNA, partial sequence	1109	1109	100%	0.0	96%

Isolate 22, Reverse Primer, sequence

TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGTGTAGCCCTACTC
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGATAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCC
CAGGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
CCCACGCTTTCGCAC

Isolate 29, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	451	451	100%	3e-127	100%
2	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	424	424	100%	6e-119	98%
3	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	418	418	100%	3e-117	98%
4	NR_042412.1	Providencia heimbachae strain : DSM 3591 16S ribosomal RNA, complete sequence	381	381	100%	3e-106	95%
5	NR_042415.1	Providencia vermicola strain : OP1 16S ribosomal RNA, complete sequence	377	377	100%	4e-105	95%
6	NR_042413.1	Providencia rettgeri strain : DSM 4542 16S ribosomal RNA, complete sequence	372	372	100%	2e-103	94%
7	NR_043750.1	Morganella psychrotolerans strain U2/3 16S ribosomal RNA, partial sequence	368	368	100%	3e-102	94%
8	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	366	366	100%	1e-101	94%
9	NR_043751.1	Morganella morganii strain DSM 14850 16S ribosomal RNA, partial sequence	357	357	100%	6e-99	93%
10	NR_024848.1	Providencia stuartii strain ATCC 29914 16S ribosomal RNA, partial sequence	355	355	100%	2e-98	93%
11	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	351	351	84%	3e-97	98%
12	NR_042053.1	Providencia alcalifaciens DSM 30120 strain CIP8290T (ATCC9886T) 16S ribosomal RNA, complete sequence	350	350	100%	1e-96	93%
13	NR_028938.1	Morganella morganii strain M11 16S ribosomal RNA, partial sequence	339	339	100%	2e-93	92%
14	NR_042811.1	Arsenophonus nasoniae strain ATCC 49151 16S ribosomal RNA, partial sequence	329	329	100%	1e-90	91%
15	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	324	324	100%	6e-89	91%
16	NR_042945.1	Xenorhabdus cabanillasii strain USTX62 16S ribosomal RNA, partial sequence	322	322	95%	2e-88	91%

Isolate 29, Forward Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
17	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	320	320	98%	8e-88	91%
18	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	318	318	100%	3e-87	90%
19	NR_037110.1	Serratia odorifera strain PADG 1073 16S ribosomal RNA, partial sequence	318	318	100%	3e-87	90%
20	NR_041975.1	Brenneria quercina strain DSM 4561 16S ribosomal RNA, partial sequence	318	318	100%	3e-87	90%
21	NR_043643.1	Xenorhabdus griffiniae strain ID10 16S ribosomal RNA, partial sequence	313	313	100%	1e-85	90%
22	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	313	313	100%	1e-85	90%
23	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	313	313	98%	1e-85	90%
24	NR_024644.1	Serratia rubidaea strain JCM1240 16S ribosomal RNA, partial sequence	313	313	100%	1e-85	90%
25	NR_026050.1	Brenneria salicis strain ATCC 15712 16S ribosomal RNA, partial sequence	311	311	100%	5e-85	90%

Isolate 29, Forward Primer, sequence

GCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAATGTCTACG
GACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGGACTGA

Isolate 29, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
1	NR_043997.1	Proteus mirabilis strain NCTC 11938 16S ribosomal RNA, partial sequence	1249	1249	100%	0.0	99%
2	NR_043998.1	Proteus penneri strain NCTC 12737 16S ribosomal RNA, partial sequence	1238	1238	100%	0.0	99%
3	NR_025336.1	Proteus vulgaris strain DSM 30118 16S ribosomal RNA, partial sequence	1210	1210	100%	0.0	99%
4	NR_043648.1	Xenorhabdus hominickii strain KE01 16S ribosomal RNA, partial sequence	1203	1203	100%	0.0	99%
5	NR_043999.1	Proteus myxofaciens strain NCIMB 13273 16S ribosomal RNA, partial sequence	1190	1190	100%	0.0	98%
6	NR_043637.1	Xenorhabdus koppenhoeferi strain USNJ01 16S ribosomal RNA, partial sequence	1182	1182	100%	0.0	98%
7	NR_042327.1	Xenorhabdus ehlersii strain :DSM 16337 16S ribosomal RNA, partial sequence	1171	1171	100%	0.0	98%
8	NR_043646.1	Xenorhabdus kozodoii strain SaV 16S ribosomal RNA, partial sequence	1170	1170	100%	0.0	98%
9	NR_042325.1	Xenorhabdus innexi strain :DSM 16336 16S ribosomal RNA, partial sequence	1166	1166	100%	0.0	98%
10	NR_043634.1	Xenorhabdus stockiae strain TH01 16S ribosomal RNA, partial sequence	1160	1160	100%	0.0	98%
11	NR_042326.1	Xenorhabdus budapestensis strain :DSM 16342 16S ribosomal RNA, partial sequence	1155	1155	100%	0.0	97%
12	NR_027194.1	Xenorhabdus japonica strain SK-1T 16S ribosomal RNA, partial sequence	1142	1142	100%	0.0	97%
13	NR_043642.1	Xenorhabdus doucetiae strain FRM16 16S ribosomal RNA, partial sequence	1136	1136	100%	0.0	97%
14	NR_025875.1	Xenorhabdus poinarii strain G1 16S ribosomal RNA, partial sequence	1136	1136	100%	0.0	97%
15	NR_042822.1	Xenorhabdus beddingii strain DSM4764 16S ribosomal RNA, partial sequence	1133	1133	100%	0.0	97%
16	NR_042820.1	Xenorhabdus bovienii strain DSM4766 16S ribosomal RNA, partial	1133	1133	100%	0.0	97%

Isolate 29, Reverse Primer							
#	Accession	Description	Max Score	Total Score	Query Coverage	E value	Max ident
		sequence					
17	NR_042328.1	Xenorhabdus szentirmaii strain :DSM 16338 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	96%
18	NR_037074.1	Photorhabdus luminescens subsp. luminescens strain Hb 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	96%
19	NR_025334.1	Obesumbacterium proteus strain 42 16S ribosomal RNA, partial sequence	1122	1122	100%	0.0	96%
20	NR_042821.1	Xenorhabdus nematophila strain DSM3370 16S ribosomal RNA, partial sequence	1120	1120	100%	0.0	96%
21	NR_026538.1	Dickeya paradisiaca strain LMG 2542 16S ribosomal RNA, partial sequence	1118	1118	100%	0.0	96%
22	NR_042411.1	Providencia rustigianii strain : DSM 4541 16S ribosomal RNA, complete sequence	1116	1116	100%	0.0	96%
23	NR_037112.1	Serratia proteamaculans strain 4364 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	96%
24	NR_041972.1	Erwinia chrysanthemi strain DSM 4610 16S ribosomal RNA, partial sequence	1116	1116	100%	0.0	96%
25	NR_041921.1	Dickeya dadantii strain CFBP 1269 16S ribosomal RNA, partial sequence	1114	1114	100%	0.0	96%

Isolate 29, Reverse Primer, sequence

TTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAG
TCGAGTTGCAGACTCCAATCCGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCATTGTAGCACGTGTGTAGCCCTACTC
GTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCACCATTACGTGCTGGCAACAAAGGATAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGGAT
GTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCC
CAGGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCACGGTTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
CCCACGCTTTCGCACCTGA

APPENDIX C

WORK INSTRUCTIONS: DWI-01

Key Words: Swab, DFU, Nucleic Acid, Microorganism

WORK INSTRUCTIONS FOR THE EXTRACTION OF MICROORGANISMS, NUCLEIC ACIDS, AND PLGA MICROSPHERES FROM ENVIRONMENTAL SAMPLES

Originated by: _____ Date _____

Stacy Dean, Research Scientist

Applied Biology and Aerosol Technology

Reviewed by: _____ Date _____

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Applied Biology and Aerosol Technology

Approved by: _____ Date _____

Chuck DeSanti, Manager

Applied Biology and Aerosol Technology

Battelle

Applied Biology and Aerosol Technology

505 King Avenue

Columbus, Ohio 43201

Work Instructions for the Extraction of Microorganisms, Nucleic Acids, and PLGA Microspheres from Environmental Samples

Scope

This Work Instruction (WI) describes the process that will be followed for the extraction of environmental samples for nucleic acids, microorganisms, and PLGA microspheres. The method outlined in this document pertains to project samples and may be altered if necessary for sample processing, but deviations from the WI will be approved by the Principal Investigator (PI) or designee prior to beginning work.

Purpose

The purpose of this document is to provide detailed instructions for the extraction of samples for further analysis of viable microorganisms, nucleic acids, and PLGA microspheres.

References

Material Safety Data Sheets (MSDS), as appropriate

Biosafety Manual, Recommended Practices for Biosafety Level 2 Agents, Building 20 Biosafety Manual, (current version)

Definitions

BSC—Biological Safety Cabinet

BSL2—Biological Safety Level 2

hsDNA—Herring sperm DNA

SDS—Sodium Dodecyl Sulfate

Procedures

Special Concerns:

All samples will be handled using aseptic technique and all manipulations will occur in a decontaminated BSC following the procedures outlined in the Building 20 Biosafety Manual for BSL-2 microorganisms.

General:

Staff members working on this effort will review this WI and program specific information not included in this WI before operations occur.

Staff members will note the locations of nearest emergency equipment, including the nearest exits, eyewash, safety shower, and fire extinguishers.

Staff members will don safety glasses and lab coats upon entering the lab. At a minimum, one pair of latex gloves or nitrile gloves will be worn for all procedures which involve handling of biological materials or chemicals. Two pairs of gloves, at least one pair nitrile, will be worn while handling reactant materials and decontamination solutions and while working in the BSC, including during the cleanup process.

Waste Disposal—strict adherence to the "Discharge to Drain" permits is required. If a Discharge to Drain permit is not in place, chemicals and media must be collected for proper disposal.

Equipment Needed:

Nitrile or latex gloves or equivalent

Disposable pipettes: 10 mL, 25 mL, sterile

Pipet Aid

Biohazard bags, various sizes

Steriflip-GP, 0.22 μm (Millipore Cat. No. SCGP00525)

Refrigerator, 4 ±3°C

Variable volume pipettes (such as Pipetman P-2, P-20, P-200, and P-1000)

Pipette tips for variable volume pipettes, aerosol resistant, sterile

Vortex

Incubator, 65 ±2°C

Biological safety cabinet, Class II

Timer

Vacuum

Materials Required:

DNA erase or equivalent

Isopropyl alcohol

10-15% bleach

1X phosphate buffered saline (PBS), pH 7.2

20% SDS

Nuclease-free water, sterile

DNA typing-grade hsDNA

250 mL bottles, sterile

2 mL polypropylene cryovial tubes

50 mL conical tubes

Procedure:

Thoroughly decontaminate the biosafety cabinet and all processing areas (bench tops, incubators, etc.) before use according to the three-step ABAT decontamination regimen. Decontaminate all samples, reagents, and other materials passed into or out of the BSC.

Collect pre-swabs according to DWI-02-01

Working in a BSC, aseptically (using sterile forceps, if needed) transfer each original sample, or portion of original sample, into a 250 mL bottle. Decontaminate gloves between samples, change gloves if they come into contact with any portion of the original samples.

Obtain a negative matrix control (one for each matrix type in the sample set) and record on Form B.

Obtain a positive matrix control (one for each matrix type in the sample set) that has been previously spiked with *B. atrophaeus* DNA and PLGA beads and record on Form B.

Add sterile 1X PBS to pre-wet each sample according to the following table:

Matrix	Pre-Wet Volume (mL)	Extraction Volume (mL)
Gauze (~58 cm^2)	5	10
Membrane Filter (<100 cm2)	2	10
Grease (1 g)	5	10
Crax (1 g)	5	10

Vortex each sample for 30 sec. to mix (record on Form A)

Add additional 1X PBS (extraction volume) and vortex each sample for 30 sec. to mix (record on Form B).

Allow samples to sit at room temperature for 15 min, vortex for 30 seconds, incubate at room temperature for another 15 min, and then vortex again for 30 seconds. Document the incubation and vortex times on Form A.

Remove a 1 mL aliquot from each sample into separate, sterile, 15 mL conical tubes. Microbiological extracts may be stored at 4 ºC for 24 h prior to analysis, if necessary.

Plate each sub-sample onto BHIA for microbial analysis (200 µL/plate).

Streak *B. atrophaeus* onto BHIA (3 or 4 phase streak) for comparison.

Record the volume remaining in the bottle on Form B.

Add 20% SDS to the remaining volume, to achieve a final concentration of 0.1% v/v.

The amount required should be calculated by converting the sample volume to microliters, then dividing by 200. For example, to calculate the amount required for a 12 mL sample:

12 mL x 1000µL/1mL = 12000 µL /200 = 60 µL 20% SDS

Add hsDNA to each sample 1 µL/mL and record on Form B.

Vortex each sample for 30 sec. to mix (record on Form A).

Incubate at 65 ±2°C for 15 min, vortex for 30 seconds; incubate an additional 15 min at 65 ±2°C, and then vortex again for 30 seconds. Document the incubation and vortex times on Form A.

Transfer the extracted volume to a 50 mL conical tube (use a pipet to squeeze as much liquid out of the matrix as possible).

Filter the extract with a Steriflip filter to remove large particles.

The filtered extract will proceed to the isopropanol precipitation procedure ABAT-V-012, and will ultimately be analyzed on the 7900HT.

Decontaminate the BSC and all processing areas.

Maintenance Procedures:

Maintenance will be performed in accordance with the individual equipment manuals or SOPs. There is no specific maintenance required for this procedure.

Emergency/First Aid Procedures:

In the event of an emergency, staff will turn off equipment as possible, evacuate the laboratory, and notify Battelle security if necessary.

First Aid/Self-Aid Procedures

If physical injuries occur, first aid or self-aid will be administered and Health Services will be called (4-4444 or 911) on internal Battelle phones.

Quality Control

Training and documentation of competency of personnel as being proficient in the use of this procedure is required.

All verifications, data, and data manipulations will be documented/recorded and available upon request by the Program Manager or Principal Investigator to facilitate review.

Forms and Attachments

Documentation of Extraction Steps

Documentation of Volumes for Extraction

Form A. Documentation of Extraction Steps

Project	**DYNAMAC**
Sample Set	
Date	

1. Remove samples from storage.....Room/Temperature_____

Record sample matrices on Form B, column A and transfer to 250 mL Corning storage bottle.

Pre-wet samples with extraction buffer, record volume of buffer added on Form B, column B. □ Vortex briefly (~30 sec.).

Add extraction buffer to samples and record volume of buffer added on Form B, column C. □ Vortex briefly (~30 sec.).

Incubate the samples at 25 ± 3°C for 15 ± 3 min. Start time: _____ End time: _____ □ Vortex briefly.

Incubate the samples 25 ± 3°C for 15 ± 3 min. Start time: _____ End time: _____ □ Vortex briefly.

Remove microbiological extract for analysis and transfer to 15 mL conical. Record on Form B, column E.

Record volume remaining for nucleic acid analysis on Form B, column F.

Add SDS to a final concentration of 0.1% v/v, record volume added on Form B, column G.

SDS Lot # _____ Expiration Date: _____

Add hsDNA (1 µL hsDNA/mL sample) , record volume added on Form B, column H.

hSDNA Lot # _____ Expiration Date: _____

Vortex briefly. Incubate the extracts at 65 ± 3°C for 15 ± 3 min. Thermometer # _____ Cal. Due _____

Actual Temperature: _____ Start time: _____ End time: _____ □ Vortex briefly.

Incubate the extracts at 65 ± 3°C for 15 ± 3 min. Thermometer # _____

Actual Temperature: _____ Start time: _____ End time: _____ □ Vortex briefly.

Transfer extracts to 50 mL conical tubes and clarify by filtering through Steriflip filter units.

Measure volumes recovered and place into pre-labeled OakRidge tube; note on Form B, column I. Proceed to Alcohol Precipitation using ABAT-V-012.

Performed by: _____ Date: _____

Reviewer: _____ Date: _____

Form B. Documentation of Volumes for Extraction

Project	**DYNAMAC**
Set Number	
Date	

Sample #	A Sample matrix	B 1 X PBS Pre-wet Vol. Added (mL)	C 1 X PBS Extraction Vol. (mL)	D Total Volume 1 X PBS Added (mL) B + C)	E Volume Extract Removed for Micro Analysis (mL)	F Volume Remaining for Nucleic acid Analysis (mL) (D – E)	G Volume hsDNA Added (µL)	H Volume SDS Added (µL)	I Volume recovered from Sterifilip Wash (mL)

Record sample notes on additional pages if necessary.

Performed by: _____ Date: _____

Reviewer: _____ Date: _____

Form C. Microbiological Analysis

Project	**DYNAMAC**
Set Number	
Date	

Sample #	**Colony Morphology (Describe: size, color, edge, shape, roughness)**				
	1	**2**	**3**	**6**	**5**
B. atrophaeus Control	2mm, orange, entire,	N/A	N/A	N/A	N/A

Example description: *B. atrophaeus* should be ~2 mm, orange, entire, raised, and smooth.

Performed by: _____ Date: _____

Reviewer: _____ Date: _____

APPENDIX C. PHOTOLOG OF PLANT ACTIVITIES

Subject: Darling International, Inc. Plant
Site: Darling International, Inc.
Photograph No.: 1 **Date:** 10/19/2010
Direction: Northeast **Photographer:** Neil Daniell

Subject: DATS collected samples from the processing building on the rendering facility.
Site: Darling International, Inc.
Photograph No.: 2 **Date:** 10/19/2010
Direction: Northeast **Photographer:** Neil Daniell

Subject: Receiving floor of Darling International, Inc. Plant. Pit is in the background.
Site: Darling International, Inc.
Photograph No.: 3 **Date:** 10/19/2010
Direction: East **Photographer:** Mike Marshall

Subject: Swab samples 1a and 1b taken from the southeast corner of the receiving floor.
Site: Darling International, Inc.
Photograph No.: 4 **Date:** 10/19/2010
Direction: West and down **Photographer:** Mike Marshall

Subject: Swab amples 2a and 2b taken from the northwest corner of the receiving floor.
Site: Darling International, Inc.
Photograph No.: 5 **Date:** 10/19/2010
Direction: Northwest and down **Photographer:** Mike Marshall

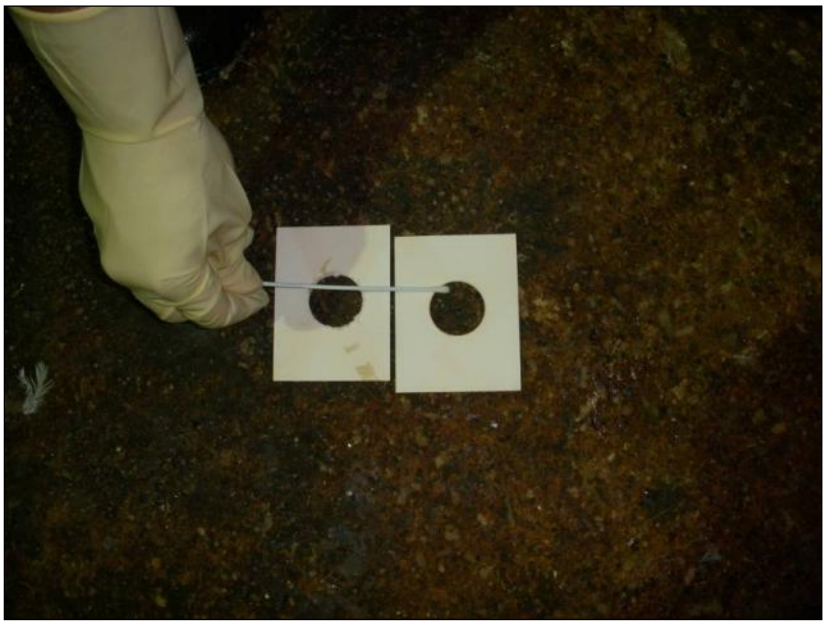
Subject: DATS collected swab amples 3a and 3b from the central vertical wall of the tipping floor pit.
Site: Darling International, Inc.
Photograph No.: 6 **Date:** 10/19/2010
Direction: West **Photographer:** Mike Marshall

Subject: Swab amples 4a and 4b taken from the northwest side (back vertical wall) of the pit.

Site: Darling International, Inc.
Photograph No.: 7 **Date:** 10/19/2010
Direction: East **Photographer:** Mike Marshall

Subject: Waste water collection sump. Swab amples 5a and 5b were collected from the foreground area (south side).

Site: Darling International, Inc.
Photograph No.: 8 **Date:** 10/19/2010
Direction: North **Photographer:** Neil Daniell



Subject: DATS collected samples 5a and 5b from the plant floor on the south side of the raw pit sump.

Site: Darling International, Inc.

Photograph No.: 9 **Date:** 10/19/2010

Direction: North and down **Photographer:** Mike Marshall

Subject: Wastewater samples 13a and 13b collected from inside the sump during the surrogate selection phase. Inset is a close-up of the collection process.

Site: Darling International, Inc.

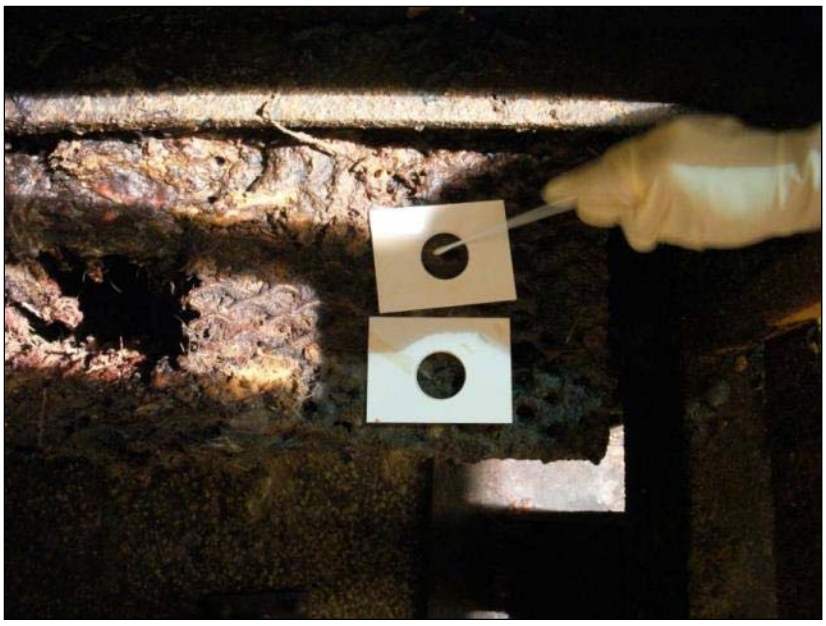
Photograph No.: 10 **Date:** 10/19/2010

Direction: North and down **Photographer:** Mike Marshall

Subject: Incline auger leading from the pit to the cooking process.
Site: Darling International, Inc.
Photograph No.: 11 **Date:** 10/19/2010
Direction: East **Photographer:** Mike Marshall

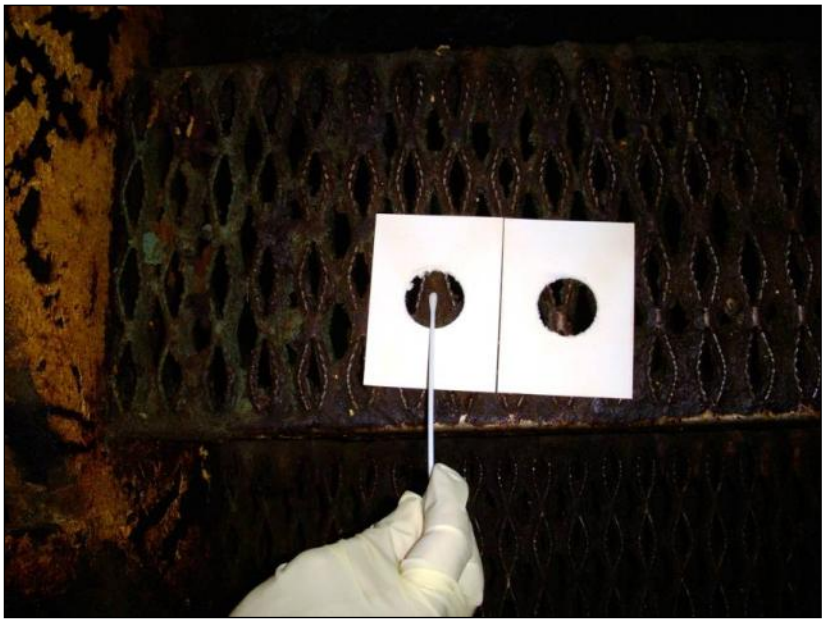
Subject: DATS collected samples 6a and 6b from the side wall of the incline auger.
Site: Darling International, Inc.
Photograph No.: 12 **Date:** 10/19/2010
Direction: South **Photographer:** Mike Marshall

Subject: Large grinder used in the rendering process.
Site: Darling International, Inc.
Photograph No.: 13 **Date:** 10/19/2010
Direction: Northeast **Photographer:** Neil Daniell



Subject: DATS collected samples 7a and 7b from the large grinder used in the rendering process.
Site: Darling International, Inc.
Photograph No.: 14 **Date:** 10/19/2010
Direction: East and down **Photographer:** Mike Marshall

Subject: Stairwell between the tallow tanks (left) and the cooker (right).
Site: Darling International, Inc.
Photograph No.: 15 **Date:** 10/19/2010
Direction: East **Photographer:** Mike Marshall



Subject: Swab samples 8a and 8b were collected from the stairwell between the tallow tanks and the cooker.
Site: Darling International, Inc.
Photograph No.: 16 **Date:** 10/19/2010

Direction: East and down **Photographer:** Mike Marshall

Subject: The load out area used by the Darling International plant.
Site: Darling International, Inc.
Photograph No.: 17 **Date:** 10/19/2010
Direction: South **Photographer:** Mike Marshall

Subject: DATS collected swab samples 9a and 9b from an undisturbed locale in the rear of the load out area.

Site: Darling International, Inc.
Photograph No.: 18 **Date:** 10/19/2010
Direction: South and down **Photographer:** Mike Marshall

Subject: The crax grinder used by the Darling International plant. Note heavy dust presences.

Site: Darling International, Inc.
Photograph No.: 19 **Date:** 10/19/2010
Direction: Northwest **Photographer:** Mike Marshall

Subject: Swab samples 10a and 10b were collected from the base (southeast corner) of the crax grinder.

Site: Darling International, Inc.

Photograph No.: 20 **Date:** 10/19/2010

Direction: Northwest and down **Photographer:** Mike Marshall

Subject: Crax grinder storage used by the Darling International plant.

Site: Darling International, Inc.

Photograph No.: 21 **Date:** 10/19/2010

Direction: Northwest **Photographer:** Mike

Marshall

Subject: DATS collected samples 11a and 11b from an undisturbed area of the crax grinder storage bins.

Site: Darling International, Inc.

Photograph No.: 22 **Date:** 10/19/2010

Direction: West and down **Photographer:** Mike Marshall

Subject: Truck receiving bay used by the Darling International plant. Swab samples 12a and 12b were collected from the background area near the drain.

Site: Darling International, Inc.
Photograph No.: 23 **Date:** 10/19/2010
Direction: Southwest **Photographer:** Mike Marshall

Subject: Samples 12a and 12b were collected the southwest side the truck receiving bay.

Site: Darling International, Inc.
Photograph No.: 24 **Date:** 10/19/2010
Direction: Southwest and Down **Photographer:** Mike Marshall

Subject: Darling International plant employees cleaning the tipping floor for the

rendering study.

Site: Darling International, Inc.
Photograph No.: 25 **Date:** 10/16/2011
Direction: Southeast **Photographer:** Neil Daniell

Subject: DATS personnel performaing background air sampling inside Darling International plant.

Site: Darling International, Inc.
Photograph No.: 26 **Date:** 10/19/2011
Direction: South **Photographer:** Neil Daniell

Subject: Air sampling pump collecting a sample from inside Darling International plant

during the rendering study.

Site: Darling International, Inc.
Photograph No.: 27 **Date:** 10/19/2011
Direction: South **Photographer:** Neil Daniell

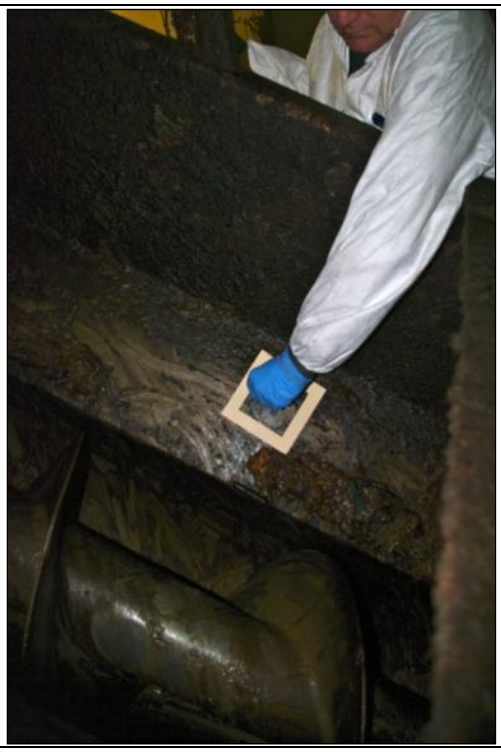
Subject: Background air sampling outside of the Darling International plant.

Site: Darling International, Inc.
Photograph No.: 28 **Date:** 10/19/2011
Direction: Northeast **Photographer:** Neil Daniell

Subject: DATS collecting a background wipe sample on the tipping floor during the final study.

Site: Darling International, Inc.
Photograph No.: 29 **Date:** 10/19/2011
Direction: East **Photographer:** Leroy

Mickelsen

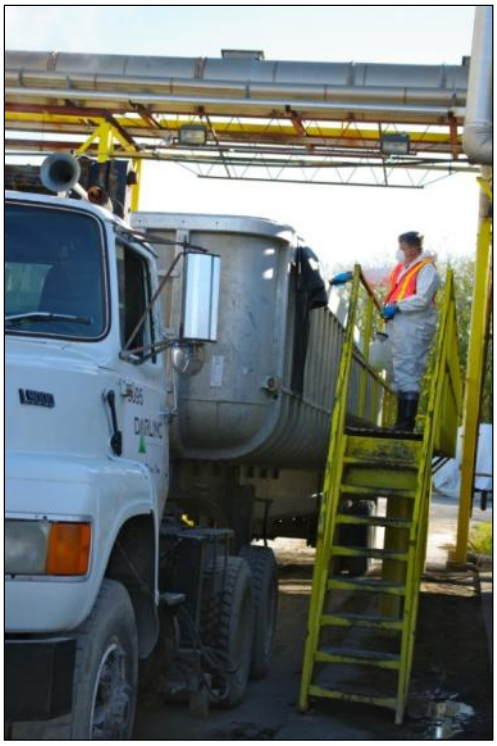


Subject: DATS collecting a background wipe sample on the wall of the auger during the final study.

Site: Darling International, Inc.

Photograph No.: 30 **Date:** 10/19/2011

Direction: South **Photographer:** Leroy Mickelsen



Subject: EPA personnel inoculating an incoming load of carcasses.
Site: Darling International, Inc.
Photograph No.: 31 **Date:** 10/20/2011
Direction: South **Photographer:** Melissa Ivancevich

Subject: Air sampling outside of the Darling International plant during processing of inoculated loads.
Site: Darling International, Inc.
Photograph No.: 32 **Date:** 10/20/2011
Direction: East **Photographer:** Melissa Ivancevich

Subject: Inoculated load of carcasses being deposited onto the tipping floor.
Site: Darling International, Inc.
Photograph No.: 33 **Date:** 10/20/2011
Direction: South **Photographer:** Melissa Ivancevich

Subject: DATS personnel collecting a wipe sampling from the tipping floor during processing of inoculated carcasses.
Site: Darling International, Inc.
Photograph No.: 34 **Date:** 10/20/2011
Direction: East **Photographer:** Melissa Ivancevich

Subject: DATS wipe sampling wall of auger during processing of inoculated loads.
Site: Darling International, Inc.
Photograph No.: 35 **Date:** 10/20/2011
Direction: East **Photographer:** Melissa Ivancevich

Subject: Cleaning of a truck using a bleach solution after it has dumped its inoculated load.
Site: Darling International, Inc.
Photograph No.: 36 **Date:** 10/20/2011
Direction: South **Photographer:** Melissa Ivancevich

Subject: Post-inoculation air sampling inside of plant.
Site: Darling International, Inc.
Photograph No.: 37 **Date:** 10/21/2011
Direction: East **Photographer:** Leroy Mickelsen

Subject: DATS post-inoculation wipe sampling of inside wall of auger.
Site: Darling International, Inc.
Photograph No.: 38 **Date:** 10/21/2011
Direction: South **Photographer:** Leroy Mickelsen

Subject: DATS collecting a sample of the tippling floor during the post-inoculation phase.

Site: Darling International, Inc.
Photograph No.: 39 **Date:** 10/21/2011
Direction: Southeast **Photographer:** Leroy Mickelsen

Subject: DATS collecting a sample from the wall of the tipping floor pit during the post-inoculation phase. Note that plant personnel frequently push material against this wall using a front loader and deposit it into the pit.

Site: Darling International, Inc.
Photograph No.: 40 **Date:** 10/21/2011
Direction: Southeast **Photographer:** Leroy Mickelsen

Appendix D – Sample Chain of Custody Sheets

USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

	Relinquished By (Date / Time)	Received By (Date / Time)
1	Anne Busher 10/22	10/22/11
2		
3		
4		

Sampler Signature:

For Lab Use Only
Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-AIR -10-19-11-ABC-B1	G	Ambient Air	START - 10:32; STOP - 12:32; FLOW - 1.058 Liter Per Minute ; Cooker Room near scrubber intake.	10/19/2011	10:32	12:32	L	Cooker Room near scrubber intake	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B2	G	Ambient Air	START - 10:39; STOP - 12:39; FLOW - 1.078 Liter Per Minute ; Window Btw Sump & Tipping Floor	10/19/2011	10:39	12:39	L	Window Btw Sump & Tipping Floor	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B3	G	Ambient Air	START - 10:44; STOP - 12:55; FLOW - 1.013 Liter Per Minute ; DAF Tank Area	10/19/2011	10:44	12:55	L	DAF Tank Area	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B4	G	Ambient Air	START - 10:47; STOP - 13:04; FLOW - 1.018 Liter Per Minute ; Soap Stock Receiving Tanks	10/19/2011	10:47	13:04	L	Soap Stock Receiving Tanks	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B5	G	Ambient Air	START - 10:52; STOP - 12:57; FLOW - 1.037 Liter Per Minute ; East of innoculating area	10/19/2011	10:52	12:57	L	East of innoculating area	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B6	G	Ambient Air	START - 10:56; STOP - 12:58; FLOW - 1.060 Liter Per Minute ; West of Innoculating Area	10/19/2011	10:56	12:58	L	West of Innoculating Area	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B7	G	Ambient Air	START- 11:03 STOP - 13:08; FLOW - 1.012 Liters Per Minute; Front Parking Lot	10/19/2011	11:03	13:08	L	Front Parking Lot	Ice Only	x	x	x
Darling International	IRP-AIR -10-19-11-ABC-B8	G	Field QC	Field Blank	10/19/2011	Field Blank	14:10	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-001	G	Ambient Air	START - 8:18; STOP - 12:18; FLOW - 1.052 Liter Per Minute ; Cooker Room near scrubber intake.	10/20/2011	8:18	12:18	M	Cooker Room near scrubber intake	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-002	G	Ambient Air	START - 10:00; STOP - 14:00; FLOW - 1.050 Liter Per Minute ; Window Btw Sump & Tipping Floor	10/20/2011	10:00	14:00	M	Window Btw Sump & Tipping Floor	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-003	G	Ambient Air	START - 10:40; STOP - 14:40; FLOW - 1.005 Liter Per Minute ; DAF Tank Area	10/20/2011	10:40	14:40	M	DAF Tank Area	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-004	G	Ambient Air	START - 14:33; STOP - 18:33; FLOW - 1.024 Liter Per Minute ; Soap Stock Receiving Tanks	10/20/2011	14:33	18:33	M	Soap Stock Receiving Tanks	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-005	G	Ambient Air	START - 8:13; STOP - 12:16; FLOW - 1.011 Liter Per Minute ; East of innoculanting area	10/20/2011	8:13	12:16	M	East of innoculanting area	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-006	G	Ambient Air	START - 8:15; STOP - 12:15; FLOW - 0.9941 Liter Per Minute ; West of Innoculating Area	10/20/2011	8:15	12:15	M	West of Innoculating Area	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-007	G	Ambient Air	START- 8:34 STOP - 12:34; FLOW - 1.014 Liters Per Minute; Front Parking Lot	10/20/2011	8:34	12:34	M	Front Parking Lot	Ice Only	x	x	x
Darling International	IRP-AIR-10-20-11-ABC-008	G	Field QC	Field Blank	10/20/2011	Field Blank	14:00	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0010	G	Ambient Air	START: 10:18 STOP: 14:18; FLOW RATE: 1.011; East side of innoculation area	10/21/2011	10:18	14:18	M	East side of innoculation area	Ice Only	x	x	x

Shipment for Case Complete? N	Sample(s) to be used for laboratory QC.	Additional Sampler Signature(s).	Cooler Temperature Upon Receipt:	Chain of Custody Seal Number:	
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ENUM = Enumeration, Fluorescen = PLGA, PCR = Bacterial ID-PCR

TR Number: 7-083090244-102211-0001

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USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
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Sampler Signature:

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Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-WIPE-10-20-11-ABC-006	G	Wipe Sample	Tipping Floor; 12 Feet from Pit Wall	10/20/2011	NA	14:28	H	Tipping Floor; 12 Feet from Pit Wall	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-007	G	Wipe Sample	Wall of Auger	10/20/2011	NA	16:42	H	Wall of Auger	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-008	G	Wipe Sample	Tipping Floor; 12 feet from pit wall	10/20/2011	NA	16:31	H	Tipping Floor; 12 Feet from Pit Wall	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-009	G	Field QC	Field Blank	10/20/2011	NA	16:45	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0011	G	Wipe Sample	PD 1 - CENTER LEFT OF TIPPING FLOOR NEAR DOOR	10/21/2011	NA	10:28	L	PD 1	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0012	G	Wipe Sample	PD 2 - GRINDER WALL; LEFT SIDE	10/21/2011	NA	10:37	L	PD 2	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0013	G	Wipe Sample	PD 3 - AUGER COVER	10/21/2011	NA	10:52	L	PD 3	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0014	G	Wipe Sample	PD 4 - Between floor drains; in front of electrical panel	10/21/2011	NA	11:15	L	PD 4	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0015	G	Wipe Sample	PD 5 - Between floor drains; in front of electrical panel	10/21/2011	NA	11:15	L	PD 5	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0016	G	Wipe Sample	PD 6 - top of steps; large grinder	10/21/2011	NA	11:26	L	PD 6	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0017	G	Wipe Sample	PD 7 - end of railroad tracks	10/21/2011	NA	11:37	L	PD 7	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0018	G	Wipe Sample	PD 8 - Three feet south of western tallow tank	10/21/2011	NA	13:54	L	PD 8	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0019	G	Wipe Sample	PD 9 - Office Door; Cooker Room	10/21/2011	NA	14:28	L	PD 9	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0020	G	Wipe Sample	PD 10 - Floor in Front of Cooker Control Panel	10/21/2011	NA	14:36	L	PD 10	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0021	G	Wipe Sample	PD 11 - Railroad Tracks next to floor drain; ~ Tank D6	10/21/2011	NA	14:44	L	PD 11	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0022	G	Wipe Sample	PD 12 - Floor approximately 1.5 feet from control panel	10/21/2011	NA	14:54	L	PD 12	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0023	G	Wipe Sample	PD 13 - Front of Crax Loadout area	10/21/2011	NA	15:07	L	PD 13	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0024	G	Wipe Sample	PD 14 - 2 feet from small crax grinder control panel	10/21/2011	NA	15:16	L	PD 14	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0025	G	Wipe Sample	PD 15 - 3 feet wall near M2 tallow tank; in walkway	10/21/2011	NA	15:23	L	PD 15	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0026	G	Wipe Sample	PD 16 - Center of doorway near M3 Tallow Tank	10/21/2011	NA	16:24	L	PD 16	Ice Only	x	x	x

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USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
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Sampler Signature:

For Lab Use Only

Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-AIR-10-21-11-ABC-0011	G	Ambient Air	START - 10:22; STOP - 14:22; FLOW - 1.042 Liter Per Minute ; Cooker Room near scrubber intake.	10/21/2011	10:22	14:22	M	Cooker Room near scrubber intake	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0012	G	Ambient Air	START - 10:43; STOP - 14:43; FLOW - 1.048 Liter Per Minute ; Window Btw Sump & Tipping Floor	10/21/2011	10:43	14:00	M	Window Btw Sump & Tipping Floor	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0013	G	Ambient Air	START - 10:33; STOP - 14:33; FLOW - 1.006 Liter Per Minute ; DAF Tank Area	10/21/2011	10:33	14:33	M	DAF Tank Area	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0014	G	Ambient Air	START - 10:30; STOP - 14:30; FLOW - 1.015 Liter Per Minute ; Soap Stock Receiving Tanks	10/21/2011	10:30	18:33	M	Soap Stock Receiving Tanks	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0015	G	Ambient Air	START - 10:37; STOP - 14:37; FLOW - 1.009 Liter Per Minute ; Near Auger and Grinder	10/21/2011	10:37	14:37	M	Near Auger and Grinder	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0016	G	Ambient Air	START - 10:47; STOP - 14:47; FLOW - 1.008 Liter Per Minute ; Front Parking Lot	10/21/2011	10:47	14:47	M	Front Parking Lot	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-0017	G	Ambient Air	Field Blank	10/21/2011	Field Blank	15:19	M	Field Blank	Ice Only	x	x	x
Darling International	IRP-AIR-10-21-11-ABC-009	G	Ambient Air	START: 10:15 STOP: 14:15; FLOW RATE: 1.017; West side of innoculation area	10/21/2011	10:15	14:15	M	West side of innoculation area	Ice Only	x	x	x
Darling International	IRP-IW-10-20-11-ABC-001	G	Field QC	Postive Control; Innoculant Water	10/20/2011	NA	16:22	H	Innoculant Water	Ice Only	x	x	x
Darling International	IRP-WIPE-10-19-11-ABC-B1	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/19/2011	NA	11:20	L	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-19-11-ABC-B2	G	Wipe Sample	Drain; middle of floor; 12 feet from new wall	10/19/2011	NA	11:33	L	Drain; middle of floor; 12 feet from new wall	Ice Only	x	x	x
Darling International	IRP-WIPE-10-19-11-ABC-B3	G	Wipe Sample	The claw; tipping room	10/19/2011	NA	11:44	L	The claw; tipping room	Ice Only	x	x	x
Darling International	IRP-WIPE-10-19-11-ABC-B4	G	Wipe Sample	Wall of Auger; Left Side	10/19/2011	NA	11:59	L	Wall of Auger; Left Side	Ice Only	x	x	x
Darling International	IRP-WIPE-10-19-11-ABC-B5	G	Field QC	Field Blank	10/19/2011	NA	14:07	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-001	G	Wipe Sample	Wall of Auger	10/20/2011	NA	10:16	H	Wall of Auger	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-0010	G	Field QC	Field Blank	10/20/2011	NA	16:50	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-002	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/20/2011	NA	10:30	H	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-003	G	Wipe Sample	Wall of Auger	10/20/2011	NA	12:16	H	Wall of Auger	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-004	G	Wipe Sample	Tipping Floor; 12 Feet from Pit Wall	10/20/2011	NA	12:30	H	Tipping Floor; 12 Feet from Pit Wall	Ice Only	x	x	x
Darling International	IRP-WIPE-10-20-11-ABC-005	G	Wipe Sample	Wall of Auger	10/20/2011	NA	14:15	H	Wall of Auger	Ice Only	x	x	x

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USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
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Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
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Sampler Signature:

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Lab Contract No:
Unit Price:
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Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-WIPE-10-21-11-ABC-0027	G	Wipe Sample	PD 17 - Walkway; 4 feet from stairs near SS5 Tank	10/21/2011	NA	16:39	L	PD 17	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0028	G	Wipe Sample	PD 18 - 12 feet from control panel; center of flexing & tanks room	10/21/2011	NA	16:47	L	PD 18	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0029	G	Wipe Sample	PD 19 - 6 inches from drain near maint. roll up door	10/21/2011	NA	16:54	L	PD 19	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0030	G	Wipe Sample	PD 20- Center of Ramp; 5 feet from door	10/21/2011	NA	17:01	L	PD 20	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0031	G	Wipe Sample	PD 21- 12 feet from wall; near floor traing (new wall near the sump)	10/21/2011	NA	17:16	L	PD 21	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0032	G	Wipe Sample	PD 22- Center of Bay Door; 5 feet from door	10/21/2011	NA	17:22	L	PD 22	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0033	G	Wipe Sample	Wall of Auger; Stage 2 Process Sampling	10/21/2011	NA	10:05	L	Wall of Auger; Stage 2 Process Sampling	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0034	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/21/2011	NA	10:18	L	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0035	G	Wipe Sample	Wall of Auger; Stage 2 Process Sampling	10/21/2011	NA	12:02	L	Wall of Auger; Stage 2 Process Sampling	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0036	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/21/2011	NA	12:12	L	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0037	G	Wipe Sample	Wall of Auger; Stage 2 Process Sampling	10/21/2011	NA	14:01	L	Wall of Auger; Stage 2 Process Sampling	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0038	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/21/2011	NA	14:13	L	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0039	G	Wipe Sample	Wall of Auger; Stage 2 Process Sampling	10/21/2011	NA	16:00	L	Wall of Auger; Stage 2 Process Sampling	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0040	G	Wipe Sample	Tipping Floor; 12 feet from pit	10/21/2011	NA	16:08	L	Tipping Floor; 12 feet from pit	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0041	G	Field QC	Field Blank B4; inside warehouse	10/21/2011	NA	11:08	L	B4	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0042	G	Field QC	Field Blank B8; inside warehouse	10/21/2011	NA	11:50	L	B8	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0043	G	Field QC	Field Blank B12; inside warehouse	10/21/2011	NA	15:00	L	B12	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0044	G	Field QC	Field Blank B16; inside warehouse	10/21/2011	NA	16:32	L	B16	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0045	G	Field QC	Field Blank B20; inside warehouse	10/21/2011	NA	17:07	L	B20	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0046	G	Field QC	Field Blank BA1; inside warehouse	10/21/2011	NA	14:06	L	BA1	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0047	G	Field QC	Field Blank BA2; inside warehouse	10/21/2011	NA	15:53	L	BA2	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0048	G	Field QC	Field Blank BTF1; inside warehouse	10/21/2011	NA	14:20	L	BTF1	Ice Only	x	x	x
Darling International	IRP-WIPE-10-21-11-ABC-0049	G	Field QC	Field Blank BTF2; inside warehouse	10/21/2011	NA	16:19	L	BTF2	Ice Only	x	x	x

Shipment for Case Complete? N	Sample(s) to be used for laboratory QC:	Additional Sampler Signature(s):	Cooler Temperature Upon Receipt:	Chain of Custody Seal Number:	
Analysis Key:	Concentration: L = Low, M = Low/Medium, H = High	Type/Designate: Composite = C, Grab = G		Custody Seal Intact? ___	Shipment Iced? ___
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USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
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Sampler Signature:

For Lab Use Only

Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-WIPE-10-24-11-ABC-050	G	Wipe Sample	Next to Yellow Pole	10/24/2011	NA	8:09	L	PC1	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-051	G	Wipe Sample	In front of Bay Door on south side	10/24/2011	NA	8:14	L	PC2	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-52	G	Wipe Sample	In center of room on metal grate	10/24/2011	NA	8:21	L	PC3	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-053	G	Wipe Sample	On ledge on north side of tipping floor	10/24/2011	NA	8:26	L	PC4	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-054	G	Wipe Sample	Outside wall of grinder on south side of tipping floor	10/24/2011	NA	8:31	L	PC5	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-055	G	Field QC	Field Blank @ PC 5; inside warehouse	10/24/2011	NA	8:36	L	PC5 - Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-056	G	Wipe Sample	Near door and below chute to pit	10/24/2011	NA	10:27	L	PC6	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-057	G	Wipe Sample	Lid	10/24/2011	NA	8:58	L	PC7	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-058	G	Wipe Sample	Pathway at top of stairs of roto strainer behind skimmer tanks	10/24/2011	NA	10:56	L	PC8	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-059	G	Wipe Sample	Middle of floor in center of room	10/24/2011	NA	10:32	L	PC9	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-060	G	Wipe Sample	Top of stairs of large grinder in auger room	10/24/2011	NA	10:47	L	PC10	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-061	G	Wipe Sample	Field Blank; inside warehouse	10/24/2011	NA	10:51	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-062	G	Wipe Sample	center of floor, 3 feet in from drain	10/24/2011	NA	10:37	L	PC11	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-063	G	Wipe Sample	In front of doorway, 1.5 feet in from drain	10/24/2011	NA	10:42	L	PC12	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-064	G	Wipe Sample	End of railroad tracks	10/24/2011	NA	11:03	L	PC13	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-065	G	Wipe Sample	1 foot in from doorway to auger room	10/24/2011	NA	11:08	L	PC14	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-066	G	Wipe Sample	Middle of room in front of D2 tank	10/24/2011	NA	11:14	L	PC15	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-067	G	Wipe Sample	Field Blank; inside warehouse	10/24/2011	NA	11:19	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-068	G	Wipe Sample	In front of bay door	10/24/2011	NA	11:25	L	PC16	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-069	G	Wipe Sample	Middle of railroad tracks, in front of Tank D6, near drain	10/24/2011	NA	11:56	L	PC17	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-070	G	Wipe Sample	In front of office door/emergency shower	10/24/2011	NA	11:32	L	PC18	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-071	G	Wipe Sample	End of cooker, to the right of the control panel	10/24/2011	NA	11:38	L	PC19	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-072	G	Wipe Sample	End of cooker, in front of bay door	10/24/2011	NA	11:45	L	PC20	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-073	G	Wipe Sample	Field Blank; inside warehouse	10/24/2011	NA	11:50	L	Field Blank	Ice Only	x	x	x

Shipment for Case Complete? N	Sample(s) to be used for laboratory QC.	Additional Sampler Signature(s).	Cooler Temperature Upon Receipt:	Chain of Custody Seal Number:	
Analysis Key:	Concentration: L = Low, M = Low/Medium, H = High	Type/Designate: Composite = C, Grab = G		Custody Seal Intact? ___	Shipment Iced? ___

ENUM = Enumeration, Fluorescen = PLGA, PCR = Bacterial ID-PCR

TR Number: 7-083090244-102211-0001

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LABORATORY COPY

USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
2			
3			
4			

Sampler Signature:

For Lab Use Only

Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-WIPE-10-24-11-ABC-075	G	Wipe Sample	In front of Raw Soapstock Tank 2, about 4 feet away	10/24/2011	NA	14:41	L	PC22	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-076	G	Wipe Sample	Back of crax load out area	10/24/2011	NA	14:53	L	PC23	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-077	G	Wipe Sample		10/24/2011	NA	14:46	L	PC24	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-078	G	Wipe Sample	1 foot from control panel	10/24/2011	NA	14:26	L	PC25	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-079	G	Wipe Sample	Field Blank; inside warehouse	10/24/2011	NA	14:31	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-080	G	Wipe Sample	In front of doorway; 20 feet in	10/24/2011	NA	14:21	L	PC26	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-081	G	Wipe Sample	In front of M1 Tank; 6 feet out	10/24/2011	NA	13:42	L	PC27	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-082	G	Wipe Sample	In front of T3 wastewater / doorway to cooker room	10/24/2011	NA	13:48	L	PC28	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-083	G	Wipe Sample	Behind DAF tank, about 6 feet from wall	10/24/2011	NA	14:15	L	PC29	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-84	G	Wipe Sample	In front of fire extinguisher; about 4 ft from wall	10/24/2011	NA	14:05	L	PC30	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-085	G	Wipe Sample	Field blank	10/24/2011	NA	14:09	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-86	G	Wipe Sample	center of floor, 5 feet from stairs	10/24/2011	NA	9:26	L	PC31	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-087	G	Wipe Sample	south of fleshing tank	10/24/2011	NA	10:04	L	PC32	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-088	G	Wipe Sample	Beside F4 tank	10/24/2011	NA	10:09	L	PC33	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-089	G	Wipe Sample	12 feet; front of fleshing tank	10/24/2011	NA	9:58	L	PC34	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-090	G	Wipe Sample	behind supm room wall next to control panel	10/24/2011	NA	10:16	L	PC35	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-091	G	Wipe Sample	Field blank	10/24/2011	NA	10:21	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-092	G	Wipe Sample	12 feet from wall; end of grate	10/24/2011	NA	9:08	L	PC36	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-093	G	Wipe Sample	2 feet in front of control panel	10/24/2011	NA	9:41	L	PC37	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-094	G	Wipe Sample	10 feet from maintenance shed	10/24/2011	NA	9:40	L	PC38	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-095	G	Wipe Sample	Center of ramp	10/24/2011	NA	9:35	L	PC39	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-96	G	Wipe Sample	Rear of bay	10/24/2011	NA	9:15	L	PC40	Ice Only	x	x	x

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USEPA Contract Laboratory Program
Generic Chain of Custody

Reference Case
Client No:
SDG No: L

Date Shipped:	10/22/2011
Carrier Name:	FedEx
Airbill:	
Shipped to:	Battelle 505 King Ave Columbus OH 43201 (513) 362-2600

Chain of Custody Record

Relinquished By	(Date / Time)	Received By	(Date / Time)
1			
2			
3			
4			

Sampler Signature:

For Lab Use Only

Lab Contract No:
Unit Price:
Transfer To:
Lab Contract No:
Unit Price:

Station Name	Sample No	Composite Grab	Matrix	Sample Comments	Sample Date	Sample Start Time	Sample End Time	Concentration	Location Name	Preservative	Analysis Name	Analysis Name 2	Analysis Name 3
											Enumeration	PLGA	Bacterial ID-PCR
Darling International	IRP-WIPE-10-24-11-ABC-097	G	Wipe Sample	Field Blank	10/24/2011	NA	9:19	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-098	G	Wipe Sample	Wall of Auger	10/24/2011	NA	8:52	L	PC Grinder Study - Auger	Ice Only	x	x	x
Darling International	IRP-WIPE-10-24-11-ABC-099	G	Wipe Sample	12 feet from wall	10/24/2011	NA	8:41	L	PC Grinder Study - Tipping Floor	Ice Only	x	x	x
Darling International	IRP-FPG-10-24-11-ABC-001	G	Grease Sample	from spigot off take	10/24/2011	NA	13:54	L	Grease Tank	Ice Only	x	x	x
Darling International	IRP-FPC-10-24-11-ABC-001	G	Crax Sample	directly from auger screw as it filled truck	10/24/2011	NA	13:59	L	Crax Load out Area	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-018	G	Ambient Air	START - 8:59; STOP - 14:59; FLOW - 1.040 Liter Per Minute ; Window Btw Sump & Tipping Floor	10/24/2011	NA	14:59	L	Station 2	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-019	G	Ambient Air	START - 8:42; STOP - 9:45; FLOW - 1.003 Liter Per Minute ; Station 5 - South of Building; East coner	10/24/2011	NA	9:45	L	Station 5	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-020	G	Ambient Air	START - 8:51; STOP - 14:51; FLOW - 1.010 Liter Per Minute ; Station 7 - Front Parking Lot	10/24/2011	NA	14:51	L	Station 7	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-021	G	Ambient Air	START - 8:40; STOP - 14:40; FLOW - 1.012 Liter Per Minute ; Station 6 -West of Innoculating Area	10/24/2011	NA	14:40	L	Station 6	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-022	G	Ambient Air	START - 9:03; STOP - 15:03; FLOW - 1.010 Liter Per Minute ; Station 4 -Wall near soap stock tanks	10/24/2011	NA	15:03	L	Station 4	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-023	G	Ambient Air	START - 8:55; STOP - 14:55; FLOW - 1.023 Liter Per Minute ; Station 8 -Near Grinder	10/24/2011	NA	14:55	L	Station 8	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-024	G	Ambient Air	Field Blank	10/24/2011	NA	15:47	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-025	G	Ambient Air	Field Blank	10/24/2011	NA	15:48	L	Field Blank	Ice Only	x	x	x
Darling International	IRP-AIR-10-24-11-ABC-027	G	Ambient Air	START - 8:48; STOP - 11:45; FLOW - 1.006 Liter Per Minute ; Station 3 - On rotary skimmer in DAF Room	10/24/2011	NA	11:45	L	Station 3	Ice Only	x	x	x

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APPENDIX E

Formulation of Fluid D

YAKIBOU, INC.

18 July 2013

Neil Daniell
Dynamac Corp
805 Streamside Dr
McDonough GA 30252

Neil:

Per your question regarding the formulation for the Fluid D (Lot # FD2841) used for your study with *Bacillus atrophaeus*, I am providing the specifics noted below. Also attached is a copy from USP 29 with the instructions for preparation of Fluid D. Essentially, Fluid D is Fluid A (dilution peptone) with added surfactants. It is used widely in microbiological labs to keep organisms suspended in solution during dilution processes, etc, as it has no deleterious effect on their viability.

Specifics for Lot FD2841:

Reagents:

0.1% (by weight) Difco Proteose Peptone #3 (Product # 211693; lot # 6086506)

0.1% (by weight) Fisher Tween 80 (lot # 952224)

Water: Triton Distilled Water, Lot 05-04-12.

Procedure:

Two grams of each of the two reagents were added to 2 liters of distilled water and stirred until completely dissolved. The pH was adjusted to 7.18 with dilute NaOH (initial pH was 5.56). The solution was then sterilized by membrane filtration.

Please contact me if additional information is needed.

Regards,

Joseph P Dalmasso
President

P.O. Box 1418, Apex, NC 27502-3418 Ph: 1-919-557-4930 Fax: 1-919-557-4931
info@yakibou.com

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