EPA/600/R-11/020 | April 2012 | www.epa.gov/ord

United States
Environmental Protection
Agency

Multimedia Sampling During the Application of Biosolids on a Land Test Site

Office of Research and Development
National Risk Management Research Laboratory

EPA/600/R-11/020
April 2012

MULTIMEDIA SAMPLING DURING THE APPLICATION OF BIOSOLIDS ON A LAND TEST SITE

by

Eric A. Foote; Carolyn M. Acheson; Edwin F. Barth; Ronald F. Herrmann; Richard C. Brenner; D. Bruce Harris; Steven J. Naber; Robert H. Forbes, Jr.; Laura L. McConnell; and Patricia D. Millner

Contract No. EP-C-05-056
Work Assignment 4-48

Contractor

Pegasus Technical Services, Inc.
Cincinnati, OH 45219

Principal Subcontractor

Battelle
Columbus, OH 43201

U.S. Environmental Protection Agency
Office of Research and Development
National Risk Management Research Laboratory
Land Remediation and Pollution Control Division
Cincinnati, OH 45268

NOTICE

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development (ORD), funded and managed the research described herein under Contract No. EP-C-05-056. It has been subjected to the Agency's peer and administrative review and has been approved for publication as an EPA document.

FOREWORD

The U.S. Environmental Protection Agency (EPA) is charged by Congress with protecting the Nation's land, air, and water resources. Under a mandate of national environmental laws, the Agency strives to formulate and implement actions leading to a compatible balance between human activities and the ability of natural systems to support and nurture life. To meet this mandate, EPA's research program is providing data and technical support for solving environmental problems today and building a science knowledge base necessary to manage our ecological resources wisely, understand how pollutants affect our health, and prevent or reduce environmental risks in the future.

The National Risk Management Research Laboratory (NRMRL) is the Agency's center for investigation of technological and management approaches for preventing and reducing risks from pollution that threaten human health and the environment. The focus of the Laboratory's research program is on methods and their cost-effectiveness for prevention and control of pollution to air, land, water, and subsurface resources; protection of water quality in public water systems; remediation of contaminated sites, sediments, and ground water; prevention and control of indoor air pollution; and restoration of ecosystems. NRMRL collaborates with both public and private sector partners to foster technologies that reduce the cost of compliance and to anticipate emerging problems. NRMRL's research provides solutions to environmental problems by: developing and promoting technologies that protect and improve the environment; advancing scientific and engineering information to support regulatory and policy decisions; and providing the technical support and information transfer to ensure implementation of environmental regulations and strategies at the national, state, and community levels.

This publication has been produced as part of the Laboratory's strategic long-term research plan. It is published and made available by EPA's Office of Research and Development to assist the user community and to link researchers with their clients.

Sally C. Gutierrez, Director
National Risk Management Research Laboratory

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ABBREVIATIONS AND ACRONYMS

ags	above ground surface
ANOVA	analysis of variance
APEs	alkylphenol and alkylphenol ethoxylates
ASTM	American Society for Testing and Materials
ATV	all-terrain vehicle
BPA	bisphenol-A
CEC	cation exchange capacity
CFR	Code of Federal Regulations
CFU	colony forming unit
DT	detection threshold
d/T	dilutions-to-threshold
DWA	Downwind Location A
DWB	Downwind Location B
DWC	Downwind Location C
EDC	endocrine disrupting compound
EPA	U.S. Environmental Protection Agency
FAME	fatty acid methyl ester
FTIR	Fourier transform infrared
GPS	global positioning system
HCA	Hierarchal Cluster Analysis
LRPCD	Land Remediation and Pollution Control Division
MANOVA	multivariate analysis of variance
MD-GC-MS	multidimensional gas chromatography/mass spectrometry
MOB	mobile sampler
MPN	most probable number
NAS	National Academy of Sciences
NCDA&CS	North Carolina Department of Agriculture & Consumer Services
NP	nonylphenol
NRC	National Research Council
NRMRL	National Risk Management Research Laboratory
OP	octylphenol
OP-FTIR	open-path Fourier transform infrared
ORD	Office of Research and Development
ORS	optical remote sensing
PBDE	polybrominated diphenyl ether
PBS	phosphate buffered solution

PCA	principal component analysis
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PFLA	phospholipid fatty acid
PFU	plaque forming unit
PO	primary objective
ppb	parts per billion
ppm	parts per million
ppmv	parts per million by volume
QAPP	Quality Assurance Project Plan
RSD	relative standard deviation
RT	recognition threshold
SAS	Statistical Analysis Software
SPME	solid-phase microextraction
SVOC	semivolatile organic compound
THB	total heterotrophic bacteria
UHP	ultra-high purity
UWA	Upwind Location A
USDA	U.S. Department of Agriculture
VHO	viable Helminth ova
VOA	volatile organic analysis
VOC	volatile organic compound
VRPM	vertical radial plume mapping
WWTP	wastewater treatment plant

ACKNOWLEDGMENTS

This report was prepared by Battelle Memorial Institute (Battelle), Columbus, OH, a subcontractor to Pegasus Technical Services, Inc. (PTSI), Cincinnati, OH, under Contract No. EP-C-05-056 between the U.S. Environmental Protection Agency (EPA) and PTSI. Stephen L. Wright served as the EPA Project Officer, and Richard C. Brenner was the EPA Work Assignment Manager. The Battelle project lead was Eric A. Foote. This project was conducted under the direction of EPA's Environmental Stressors Management Branch (Branch Chief, Laurel J. Staley) of the Land Remediation and Pollution Control Division (LRPCD), National Risk Management Research Laboratory (NRMRL).

This project was carried out at the Piedmont Research Station of the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) in Salisbury, NC. NCDA&CS staff generously provided invaluable assistance to the researchers by making space available in their buildings and facilities, supplying logistics support, lending tools and equipment, preparing (mowing) the application field for receiving biosolids, consulting with research staff on equipment deployment, and courteously responding to many questions and requests from the research team. Without the cooperation and assistance of Piedmont Research Station Managers Joe Hampton and Ray Horton and their capable staff, this project would not have been possible. Their service is commended, and their contributions are greatly appreciated.

This project integrated research from several disciplines to evaluate the effects of land application of biosolids on air and volatile emissions and soil microbial characteristics. Measurements included chemical, physical, and microbiological analytes. Contributors to the integrated project planning included Eric A. Foote from Battelle; Carolyn M. Acheson, Edwin F. Barth, Richard C Brenner, Paul R. de Percin (retired), D. Bruce Harris (retired), Ronald F. Herrmann, Mark C. Meckes, James E. Smith, Jr. (retired), Laurel J. Staley, Stephen L. Wright, and Lawrence D. Zintek from EPA; and Laura L. McConnell and Patricia D. Millner from the U.S Department of Agriculture (USDA).

The following authors, utilizing their experience and expertise, analyzed, organized and interpreted the large quantity of data collected during this study and assessed the peer-reviewed literature for its relevance to this project. The technical interpretations of this report are based on their efforts, which are gratefully acknowledged.

Executive Summary – Eric A. Foote (Battelle), Carolyn M. Acheson (EPA), and Richard C. Brenner (EPA) with assistance from Laurel J. Staley (EPA), Mathew Morrison (previously with EPA), and James E. Smith, Jr. (EPA-retired)

Sections 1, 2, 3, and 4 – Eric A. Foote (Battelle) and Richard C. Brenner (EPA) with assistance from James E. Smith, Jr. (EPA-retired)

Section 5 – Edwin F. Barth (EPA), Eric A. Foote (Battelle), and Patricia D. Millner (USDA) with assistance from Richard C. Brenner (EPA)

Section 6 – Edwin F. Barth (EPA), D. Bruce Harris (EPA-retired), Eric A. Foote (Battelle), Laura L. McConnell (USDA), and Robert H. Forbes, Jr. (CH2M Hill) with assistance from Richard C. Brenner (EPA) and Eben D. Thoma (EPA)

Section 7 – Carolyn M. Acheson (EPA), Ronald F. Herrmann (EPA), Eric A. Foote (Battelle), and Steven J. Naber (Battelle) with assistance from Richard C. Brenner, Tracy A. Dahling, Sally J. Stoll, Troy J. Strock, Stephen L. Wright, and Lawrence D. Zintek from EPA; Melody J. Graves-Allen from Battelle; and J. Lee Heckman, M. Jacqueline Tompkins, and Susan S. VonderHaar from Shaw Environmental and Infrastructure

Section 8 – Carolyn M. Acheson (EPA), Richard C. Brenner (EPA), and Eric A. Foote (Battelle)

The Quality Assurance Project Plan (QAPP) was prepared by the following Battelle staff: Eric A. Foote (leader), Melody J. Graves-Allen, Gregory L. Headington, Shane S. Walton, Steven J. Naber, and Michael W. Holdren. The QAPP was reviewed by several EPA and USDA staff under the guidance of LRPCD Quality Assurance Manager Scott A. Jacobs, who was responsible for final approval, quality assurance (QA) auditing, QA oversight in the field, and QA review of work products such as this report.

A variety of field preparation activities including, among others, site layout, sampling and auxiliary equipment fabrication and assembly, preliminary characterization sampling and analysis, and temporary on-site trailer and laboratory deployments were critical to the conduct of this project. These activities involved many individuals from the staffs mentioned above. Special thanks are due to Gregory Headington, Shane S. Walton, and Melody Graves-Allen from Battelle; Tracy A. Dahling, Paul T. McCauley, Kim A. McClellan, and Sally J. Stoll from EPA; Patricia D. Millner from USDA; and Susan Boutros from Environmental Associates, Ltd.

Battelle staff organized and directed the sequence of events necessary to carry out this intensive week-long field effort as well as the pre- and post-post application soil sampling studies. Approximately 50 individuals from several organizations were involved on site in some capacity in this complex, multi-faceted project. The contributions of the following Battelle personnel are acknowledged and appreciated in orchestrating the orderly conduct of the many tasks required to bring this field study to a successful conclusion: Eric A Foote (project leader), Gregory L. Headington (field staff leader), Melody J. Graves-Allen, Shane S. Walton, Gary B. Carlin (Safety Officer), Hali L. Jackson, Lincoln G. Remmert, Jan R. Satola, and R. Michael Woolfe.

Vital program support and advice have been provided by EPA senior leadership from the outset of this project in dealing with policy issues that evolved during the planning and implementation stages of the multi-discipline effort. The cooperation and support of the following EPA administrative personnel are sincerely appreciated and acknowledged: Sally C. Gutierrez (Director, NRMRL), Hugh McKinnon (retired Acting Director, NRMRL), Lawrence W. Reiter (former Acting Director, NRMRL), Benjamin L. Blaney (retired Assistant Laboratory Director for Water, NRMRL), Robert A. Olexsey (retired Director, LRPCD, NRMRL), Annette M. Gatchett (former Acting Director, LRPCD, NRMRL), Patricia M. Erickson (former Acting Director, LRPCD, NRMRL), Robert K. Bastian (Office of Wastewater Management, Office of Water), Richard R. Stevens (Office of Science and Technology, Office of Water), and Patricia L. Schultz (retired Chief, Cincinnati Technical Communications and Outreach Staff).

The draft report for this project was subjected to external peer review by three acknowledged experts in land application of biosolids and bioaerosol and soils sampling methods and approaches. These three scientists were Dr. Charles Gerba, University of Arizona; Mr. Greg Kester, California Association of Sanitation Agencies; and Dr. Jordan Peccia, Yale University. Solicitation of reviews and collection and submittal of review comments were conducted under Contract with Versar, Inc., Springfield, VA via Contract No. EP-C-07-025, Task Order 72. Coordination of activities with Versar and oversight of staff review of, response to, and reconciliation of peer review comments was carried out under the leadership of EPA Branch Chief Laurel J. Staley.

In order to ensure community input to the development and conduct of this project, multiple agency personnel acted as liaisons with the public through the Information Sharing Group (ISG). The ISG's primary areas of interest have revolved around the potential impact of biosolids land application on adjacent neighbors and landowners and methods chosen by the research team to evaluate the capture, detection, identification, and quantification of chemical and biological constituents of biosolids released to the environment. The ISG has been invited to review and comment on important documentation produced during this study including the QAPP, data summaries, draft copies of the Executive Summary from this report, and finally this report in its entirety. The comments provided by the ISG have been

utilized, where appropriate, to improve the overall objectivity of and scientific approach to carrying out this project.

The ISG was co-founded by John Walker (retired Biosolids Team Leader, EPA, Office of Wastewater Management, Office of Water), Richard Giani (former Biosolids Coordinator, Pennsylvania Department of Environmental Protection [PADEP]), and Patricia D. Millner (USDA). John Borland (PADEP) provided technical and administrative assistance during the formative stages of the ISG. Laurel J. Staley served as the EPA technical liaison with the ISG throughout the lengthy process of project and work plan development, field study implementation, and report preparation and review. The collaborative efforts of the following ISG members are acknowledged with appreciation: Ned Beecher (ISG Coordinator, North East Biosolids and Residuals Association), Tom Albert (veterinarian and private citizen, Laurel, MD), Ellen Harrison (retired, Cornell Waste Management Institute), Frank Hearl (Center for Disease Control, NIOSH), Ronald Liebert (California Farm Bureau Federation), Albert Medvitz (rancher and private citizen, Rio Vista, CA), Christopher Piot (District of Columbia Water and Sewer Authority), Jay Snider (Wastewater Treatment Plant Operator, Borough of Ephrata, PA), and Henry Staudinger (lawyer and Citizen's Representative of Virginia Biosolids Use Advisory Committee).

EXECUTIVE SUMMARY

The goal of this research study was to evaluate air and soil sampling methods and analytical techniques for commercial land application of biosolids. Biosolids were surface applied at agronomic rates to an agricultural field. During the period of August 2004 to January 2005, 35 groups of analytes were measured using 13 sampling techniques. Several analytes were measured in more than one matrix. For example, fecal coliforms were measured in biosolids, air, and soil. In total, 49 analyte-matrix combinations were measured. The multimedia approach and numerous analyte-matrix combinations are unique for a field study on the land application of biosolids. For 27 combinations, data met quality criteria, and interpretation used conventional methods. Quality assurance (QA) criteria were not met, or QA data were not reported for 12 combinations. The interpretation of these data sets was affected by QA limitations, and conclusions from these data are more uncertain. No detections were observed for 10 microbial analytes. It is not clear if organisms were present but not detected or were absent.

In this study, odors were detected in the air, and chemicals and microbes were measured in the soil after land application of biosolids. Odors had dissipated after 4 days. In shallow soils, most microbial and chemical analytes remained elevated for the remainder of the study, 98 days. The conclusions of this study may have been affected by the biosolids applied, weather conditions during and after application, and other site-specific variables. Additional studies would be useful to determine if these observations are consistent with other biosolids applications. Based on the results of this study, the 27 analyte-matrix combinations yielding usable data have been demonstrated at field scale and could be used in future research within the QA context of this study. For the other analyte-matrix combinations, additional QA samples, screening of analytical labs for compliance with QA, and continued methods development are needed. This research, in combination with the work of others, may result in the development of an integrated, multimedia protocol for use in field sampling of biosolids land application.

Background

In the United States, about 60% of biosolids, the solid residues produced by wastewater treatment, are applied to land as an agricultural amendment. Many believe that biosolids application is a beneficial use of this material. In 1993 under the Clean Water Act, the U.S. Environmental Protection Agency (EPA) issued regulations governing land application of biosolids, commonly referred to as the Part 503 Rule. Biosolids are defined as sewage sludge that has been treated to meet federal and state regulations for land application. In the years since the regulations were issued, wastewater treatment technologies and practices have changed and public concerns about the land application of biosolids have grown. In 2002, the National Research Council of the National Academy of Science issued a report entitled: *"Biosolids Applied to Land: Advancing Standards and Practices"* (NRC, 2002). The report noted that no scientific evidence documented that the Part 503 Rule had failed to protect public health and recommended additional scientific work to reduce uncertainties about the potential for adverse human health effects from exposure to biosolids.

Motivated by this report and other research questions, a collaborative research team under the leadership of the EPA's Office of Research and Development was assembled. A field-scale land application study was undertaken to evaluate sampling methods and analytical techniques. The major objective of this study was to screen many of the available methods for applicability and included: four environmental matrices (air emissions, airborne particles, soil, and biosolids); 35 analyte groups; and 13 sampling methods. Air samples were measured before, during, and after application for volatile compounds, odorants, microorganisms, and endotoxins as well as their short-range transport. Airborne particulate levels were monitored. Microbial and chemical concentrations were determined for soil samples before and after biosolids application.

Study Design

This study was conducted at the North Carolina Department of Agriculture and Consumer Services Piedmont Research Station in Salisbury, NC. Class B biosolids were surface applied to a fescue field in a 100-m diameter circle by a commercial side discharge manure spreader at a target rate of 10 wet tons/acre. Biosolids had not been applied to this land previously. Monitoring began before application in August 2004 and continued through January 2005.

The sewage sludge used in this study was anaerobically digested, dewatered by centrifugation, and treated with lime. Polymer was added during sludge treatment. This type of sludge treatment is commonly used in wastewater treatment plants and is likely to produce biosolids with detectable odors and generate aerosolized particulates via surface drying, flaking, and wind erosion during land application. However, the biosolids delivered to the site were sticky, cohesive, and tended to rapidly settle onto the ground in agglomerated clumps. This characteristic visually impacted the distribution of the biosolids on the soil surface and may have introduced unplanned variance into soil/biosolids sample collection.

Key Research Results

Biosolids

The biosolids used in this study had a solids content of 28% and contained 2.3×10^9 colony forming units (CFUs) of fecal coliforms/g dry weight (gdw) total solids and 6.33 most probable number (MPN) *Salmonella* spp./gdw total solids. Several microbial characterizations were measured including total heterotrophic bacteria (THB) at 1.6×10^{11} CFUs/gdw total solids, *Escherichia coli* at 4.35×10^7 MPN/gdw total solids, total coliforms at 1.4×10^9 CFUs/gdw total solids, and *Enterococcus* spp. at 8.2×10^5 CFUs/gdw total solids. Samples were analyzed for *Staphylococcus aureus*, but none were detected.

Airborne Particles

Airborne particles were collected using impingers, impactors, and GRIMM samplers. These samples were analyzed for microbial analytes and particulate mass. Two types of microbes, THB and fungi, were detected during both the control trial and the biosolids application test, especially at sampling points near the spreader. However, no specific bacterial pathogens (i.e., *E. coli*, *Salmonella* spp., S. *aureus*, *Clostridium perfringens*, and *Enterococcus* spp.), indicator microorganisms (i.e., fecal coliforms and coliphage), or enteric viruses were detected. Organisms may have been present but not detected. Standardized and more robust bioaerosol samplers and field QA samples may clarify this question. Bacterial endotoxin samples were collected; however, due to operational and QA problems, data were unusable. The mass of particulates ≤ 5.0 μm was statistically similar in samples collected immediately before and during biosolids application. Based on these results, the mass of bioaerosol particles was not changed during biosolids land application.

Air

Odors were monitored in the field for up to 4 days following application using Nasal Rangers®, flux chambers, and off-site odor analysis. Nasal Rangers® are useful for detecting odors compared to background levels; they do not identify specific chemicals. In the near-field application area, Nasal Rangers® detected odor at approximately twice background levels for up to 2 days after application. By Day 4, odors were not detected in the near field. Odor analysis was also conducted by sampling the exhaust vents of flux chambers using Nasal Rangers® and off-site analysis by an odor panel. The flux chamber temperatures were higher than ambient temperatures due to radiant heating. This situation was

not anticipated and may have compromised observations. Exhaust gas resulted in Nasal Ranger® odor detections at about twice background on Day 1, which dissipated to background levels on Day 2. Off-site odor panel analysis resulted in an approximate five-fold increase from control levels to levels immediately following application. Comparing the results for the control trial to 22 hr after application, a total increase of about 50-fold was observed by the off-site odor panel. Concurrent with increased temperatures, elevated levels of volatile organic sulfur compounds were observed in flux chamber samples and may explain the increased odor observations.

Chemical measurements were made in conjunction with odor monitoring and included Draeger tubes (for ammonia), a Jerome analyzer (for hydrogen sulfide), and vertical radial plume mapping analysis using open-path (OP) Fourier transform infrared (FTIR) spectrophotometry. For all air samples, ammonia was measured at less than 1 ppm ammonia by Draeger tubes. Near-field OP-FTIR plume mapping following application showed an exponentially decreasing emission flux rate, initially detected at 0.063 g/s and completely dissipating by 22 hr after application. Hydrogen sulfide concentrations were similar during the control trial and biosolids application days. Samples of flux chamber exhaust from the day of biosolids application contained 15 ppmv and 0.16 ppmv of ammonia and hydrogen sulfide, respectively. Flux chamber samples for other days were below detection for ammonia and hydrogen sulfide.

Soil

Soil samples were collected prior to biosolids application and for 4 months following application. Soil measurements included: fecal coliforms as an indicator of pathogenic organisms; phospholipid fatty acids to characterize the total biomass present as well as the microbial community structure; alkylphenol ethoxylates (APEs) and metabolites such as octylphenol (OP) and nonylphenol (NP) that are potential endocrine disrupting chemicals; and soil toxicity to plants and earthworms. Soil was sampled as deep as 25 cm for several analytes, but at this site, the 0-5 cm depth proved to be the most important in understanding the potential effects of biosolids. Supporting information such as soil agronomic, temperature, biosolids distribution, and weather data were also gathered.

Supporting information is useful in placing results from this study in the context of similar research. For this study, rainfall was plentiful. As a result, soil was near moisture holding capacity throughout the study, and desiccation was unlikely to affect soil microbes. Soil temperatures varied over the sampling period from as high as 28 °C in August to 3°C in January. It is possible that cooler temperatures, especially during the last month of the study, affected observations. Reduced microbial die-off and lower aerobic degradation rates are often observed at lower temperatures and high moisture levels. Measuring the quantity of biosolids after application was an easy method to document the application rate, 7.3 to 9.5 wet tons/acre or 1.7 to 2.2 dry tons/acre, which was close to the planned rate.

At this site, land application of biosolids altered microbial and chemical soil concentrations at shallow depths. Total biomass, fecal coliforms, and NP and OP displayed increases following application that generally persisted for the 98-day post-application soil sampling period. Total biomass increased by a factor of two. Fecal coliform measurements exhibited an increase of more than 100-fold between pre- and post-application samples. However, because the laboratory reported semi-quantitative results for 48% of the samples, this finding is uncertain. Although APEs were not detected in the soil at any time, after biosolids application, the metabolites OP and NP were detected at median concentrations of 5,400 and 215 μg/kg dry solids, respectively.

Some measurements showed transient changes or no change after biosolids application. For example, the microbial community changed initially after application but returned to its pre-application structure within 28 days. Enteric viruses, *Salmonella* spp., and viable Helminth ova were observed in the

biosolids and in 20% of the soil samples throughout the study. Following biosolids application, soil toxicity exhibited no changes in earthworm mortality and seed germination while root length data were different for the two species tested. Since a limited set of ecotoxicity assays was used, it is difficult to draw broad conclusions from this dataset.

Recommendations

Additional studies would be useful to determine if the discussed observations are consistent with other types of biosolids, especially considering their nature and composition; site specific factors including soil type, types of plants, and ambient bioaerosol levels; and differing weather conditions such as wind directions and speeds. OP-FTIR techniques were useful for tracking the extent and longevity of the plume. For future air sampling, data interpretation would benefit from an experimental plan that made greater use of particulate matter sampling, meteorological monitoring, and air dispersion modeling. In addition, the collection and operational methods used to sample bioaerosols in this study would benefit from advancements such as the use of indicator organisms for positive controls and improved collection of sensitive organisms. Due to radiant heating, unanticipated elevated temperatures were observed in the flux chambers and compromised the volatile chemical and odor data. Improved flux chamber methods are needed to produce data representative of field conditions. Future soil sampling efforts should evaluate longer sampling periods and expand the chemical analyte list. Improved sampling procedures, such as normalizing concentration with a biosolids-specific chemical, may reduce sample-to-sample variability and thus increase confidence in conclusions. Pre-screening of labs analyzing microbial samples to demonstrate QA compliance would be useful for future studies.

1.0 PROJECT DESCRIPTION AND OBJECTIVES

1.1 Background and Introduction

The historical approach taken by the U.S. Environmental Protection Agency (EPA) in managing municipal wastewater treatment plant residuals or sewage sludge is largely based on the 1979 Regulation: *Criteria for Classification of Solid Waste Disposal Facilities and Practices* (EPA, 1979) as modified by the 40 CFR Parts 257, 403, and 503 Rule(s): *Standards for the Use or Disposal of Sewage Sludge* (EPA, 1993). The Part 503 Rule specifies standards for the treatment of municipal sewage sludge to be applied on land. When sewage sludge has been treated to meet federal and state regulations for land application, the resulting material is commonly called biosolids. The Part 503 Rule sets limits for land application of biosolids based on metals concentrations and/or loadings, disinfection for reduction of pathogens, and vector attraction reduction (for example, volatile solids destruction for anaerobically digested sludge).

In the years following issuance of the Part 503 Rule, the land application of biosolids has become a subject of controversy. While some view this practice as a beneficial use of biosolids, others are concerned by the practice. Anecdotal reports of illness from residents near some biosolids land application sites have been made. At the request of EPA, the National Research Council (NRC) of the National Academy of Science evaluated regulatory requirements and non-regulatory measures with respect to land application of biosolids and provided an independent review of the technical basis of the chemical and microbial contaminant regulations for biosolids land application as it pertains to human health. In July 2002, NRC completed an 18-month study and issued a report entitled *Biosolids Applied to Land: Advancing Standards and Practices* (NRC, 2002). NRC did not investigate individual reported health incidents as part of its study. They did search the published scientific literature for evidence of human health effects related to biosolids and found that no scientific studies of the alleged health incidents had been published at the time of the review. Hence, in the "Overarching Findings" section of their report, the NRC team stated: **"**There is no documented scientific evidence that the Part 503 Rule has failed to protect public health. However, additional scientific work is needed to reduce the persistent uncertainty about the potential for adverse human health effects from exposure to biosolids. There have been anecdotal allegations of disease, and many scientific advances have occurred since the Part 503 Rule was promulgated. To assure the public and protect public health, there is a critical need to update the scientific basis of the Rule to: 1) ensure that the chemical and pathogen standards are supported by current scientific data and risk assessment methods, 2) demonstrate effective enforcement of the Part 503 Rule, and 3) validate the effectiveness of biosolids-management practices" (NRC, 2002).

After careful study of the NRC report and current regulations, and with input from all relevant stakeholders, EPA responded with a 14 Point Action Plan that had a goal of strengthening the beneficial use and disposal program for municipal wastewater treatment plant residuals (Smith and Stevens, 2010). The Action Plan (http://federalregister.gov/a/03-32217) included such activities as: developing methods for microbial pollutants such as *Ascaris* ova, viruses, fecal colifroms, and Salmonella; developing analytical methods for pharmaceuticals and personal care products; conducting a targeted national sludge survey for pollutants in biosolids; conducting field studies applying biosolids to land; participating in meetings on incident tracking, exposure measurement, and sustainable land application; reviewing the criteria for molybdenum in land-applied biosolids; and assessing available tools and methodologies for conducting microbial risk assessments on pathogens. EPA has made significant progress on many Action Plan activities and continues to address these and other activities.

This report details a field research project designed to: 1) evaluate multimedia sampling methods and techniques prior to, during, and following the application of biosolids to agricultural grassland; and 2) address one of the activities (Field Studies of Application of Treated Sewage Sludge) from EPA's

Action Plan response to the NRC report. The multimedia sampling techniques included methods for measuring various components contained in the applied biosolids, entrained in aerosol emissions, discharged into the air as volatile and semi-volatile gases and odorants, and collected from the soil surface and subsurface of the applied grassland. This report documents the results of this study. This research was not designed to investigate health-related incidents and, therefore, does not constitute a health effects research study.

This report documents the approach, methodologies, results, and interpretation of a collaborative research study conducted by EPA's Office of Research and Development (ORD), National Risk Management Research Laboratory (NRMRL), Land Remediation and Pollution Control Division (LRPCD); the U.S. Department of Agriculture (USDA); the North Carolina Department of Agriculture and Consumer Services (NCDA&CS); Battelle; and other supporting groups and organizations. The target audience for this report includes EPA's Office of Water; Regional and State Biosolids Coordinators; and engineers, scientists, and consultants active in the wastewater treatment field, as well as non-traditional stakeholders such as citizens' groups.

The study began in autumn, a typical application time for biosolids in the Southeastern portion of the United States. Routine agronomic practices were utilized. In addition to evaluating sample collection and analysis methodologies, related environmental and other conditions associated with the test application were measured and/or monitored.

1.2 Project Goal

The goal of this research study was to investigate air and soil sampling methods and approaches. Ultimately, this research along with the research of others may lead to the development of a protocol that could be used in future studies to obtain data on the release of airborne and soil-bound contaminants during the application of biosolids on land. The air was sampled for selected constituents of particulates, microbes, and volatile compounds. Air particulate samples were analyzed for endotoxin. Air samples were tested for indicator organisms and several pathogens and volatile compounds including malodorants. The biosolids applied and the soil to which they were applied were also analyzed.

The study measured air emissions, their short-range transport, and soil microbial concentrations at and around the test site during biosolids application, with a focus on qualitative and quantitative characterization of the items described above. Soil microorganisms were evaluated based on the general community and specific classes including fecal coliforms, *Salmonella* spp., viable Helminth ova (VHO), and enteric viruses. Microorganisms enumerated from biosolids and air samples included fecal coliforms, total heterotrophic bacteria (THB), *E. coli*, *Salmonella* spp., *Enterococci*, *Staphylococcus aureus*, *Clostridium perfringens*, male-specific coliphage, and enteric viruses.

The multimedia approach that was used for the collection and analysis of air emissions at this test site was unique in comparison with other projects in this area of study, i.e., others focus on one or more components in individual classes of emissions (microorganisms, volatile organic compounds [VOCs], or odors) independently of each other. Data gained from this project constitute a landmark set of simultaneous multimedia information (qualitative and quantitative) associated with the application of biosolids on land and will be used to further development of method protocols for sampling at other land sites where biosolids are applied.

1.3 Project Objectives

To achieve the goal stated in Section 1.2, the research team implemented three discrete tasks in the field as described below, each with project-specific objectives. For each of these tasks, a primary objective (PO) was identified.

1.3.1 Task 1. Bioaerosol and Particulate Matter Sampling. Selected bacteria, viruses, bacterial endotoxins, and particulates were analyzed in samples taken of aerosol emissions from the biosolids pile and from the field prior to, during, and after biosolids application. Section 5.0 describes in detail the microorganisms monitored and the particulate matter analyses conducted. The PO for bioaerosol sampling was to characterize the type and concentration of the suite of viable bioaerosol components (seven bacteria, culturable enteric viruses, endotoxin, and male-specific coliphage) emitted and transported to several downwind sampling stations.

1.3.2 Task 2. Volatile Organic Compound (VOC) and Odor Sampling. A select group of VOCs and odorous compounds was monitored in emissions emanating from the application area prior to, during, and following the application of biosolids. These compounds were measured using a variety of instrumentation. Section 6.0 describes in detail the measurements of VOCs and odorous compounds. The PO for Task 2 was to determine the presence and concentration of selected VOCs and odorants in samples collected during field application, within the application area, and downwind of the application area.

1.3.3 Task 3. Land Sampling. The PO of the land sampling effort was to measure the concentration of microorganisms in biosolids applied to land in the test site and soil directly below the biosolids over time and to screen the toxicity of the biosolids. In addition, the soil concentrations of nonylphenol ethoxylates, nonylphenol, octylphenol, and Bisphenol A (BPA) were also measured. In Section 7.0, a detailed description of the land sampling methods and analytes used to accomplish the PO is available.

2.0 EXPERIMENTAL APPROACH AND TEST SITE SETUP

The overall test site design and approach used in setting up the field experiments are described in this section along with the details of the site layout and the sampling techniques employed in the field.

2.1 Experimental Approach

This research was conducted on the property of the NCDA&CS Piedmont Research Station in Salisbury, NC. The NCDA&CS provided land, personnel, and facilities in assisting with this research effort.

The test site was designed to support the eventual development of a field sampling protocol by: 1) gathering data on the emission concentrations of chemical and biological parameters prior to, during, and after biosolids land application at various upwind and downwind locations on the site, and 2) monitoring the persistence of selected microorganisms and chemicals on land. Atmospheric conditions were recorded during the application process as they were expected to have an impact on emission dynamics. Airborne microbes and particulates that may be associated with the application of biosolids were collected in bioaerosol samples.

For the purposes of this study, a 100-m diameter circle (~2-acre area) served as the focal point for this research and the location where the biosolids were applied. Various sampling and monitoring activities were conducted prior to, during, and after the application. These activities included:

- Bioaerosol monitoring
- VOC sampling including the measurement of emissions using flux chambers; open-path Fourier transform infrared (OP-FTIR) spectrometer measurements; optical remote sensing (ORS); and the determination of ammonia, hydrogen sulfide, and odorant concentrations
- Land sampling activities including physical/chemical soil properties analysis, specific chemical analysis, microbiological analysis, and ecotoxicity testing.

Hydrogen sulfide and ammonia measurements were conducted with hand-held monitors, and odorants were monitored using Nasal Rangers® and in-lab analyses of vapors captured during flux chamber sampling. These measurements, along with the bioaerosol monitoring program, were conducted within and around the 2-acre area. Land sampling activities were only conducted within the 2-acre area where biosolids had been applied. An aerial view of the test site and application area is shown in Figure 2-1. This figure also denotes the global positioning system (GPS) locations of the various sampling deployments and sample collection areas within and immediately adjacent to the application area (identified by the circle).

Semivolatile organic compounds (SVOCs) were monitored because of the speculation that many chemicals are released into a wastewater treatment facility's collection system that could potentially accumulate in the biosolids. For example, organic compounds such as brominated flame-retardants have been found to leach from biosolids into the environment (Anderson and MacRae, 2006). Inorganic compounds, such as ammonia and hydrogen sulfide, are also commonly found in biosolids and were, therefore, monitored during this study.

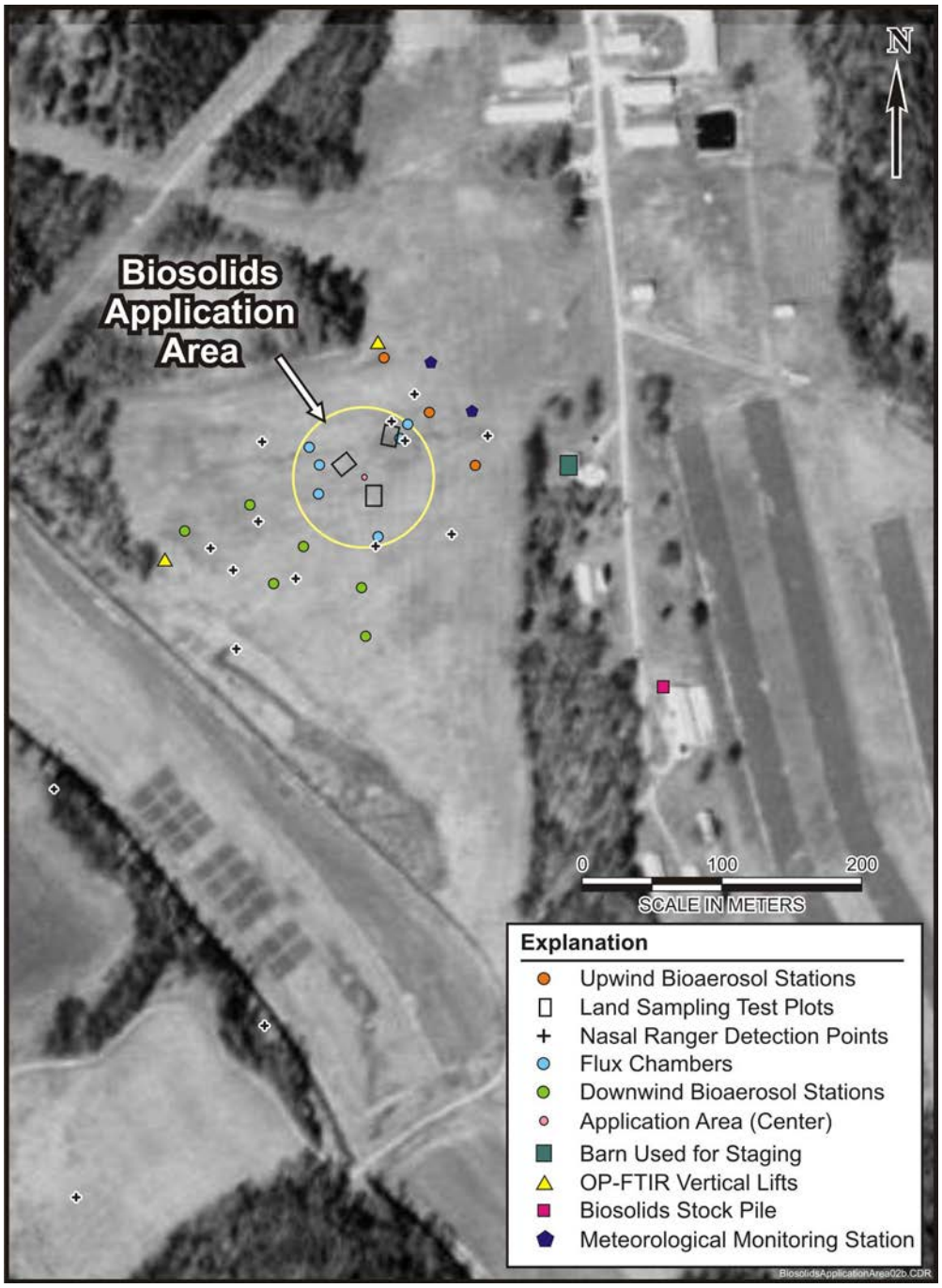


Figure 2-1. Aerial View of the Test Site and Application Area

Total particulates were monitored because of the possibility that small particulates from biosolids material may be suspended in the air during land application. It has been speculated that adjacent landowners can come in contact with these particles as they are transported off site (downwind).

In addition, it has also been speculated that pathogens, viruses and endotoxins can be adsorbed onto suspended particulates and perhaps come into contact with the nearby human population; therefore,

emissions were sampled using an array of bioaerosol sample collection equipment upwind and downwind of the experimental test site prior to and during the application of the biosolids.

Testing for odors and their intensities occurred prior to, during, and up to 2 days after the application of biosolids using hand-held olfactometers. In addition to these on-site analyses, in-lab analyses of vapor emissions captured during flux chamber sampling were analyzed to determine odor threshold.

2.2 Site Design

The test site was designed around a 100-m diameter circle so that an array of upwind and downwind sampling units could be moved (see Figure 2-2) around the circle during sampling to accommodate a major shift in wind direction should one occur. Sampling locations were positioned on four discrete lines that ran parallel to each other. The upwind line, designated UWA (Upwind Location A), was positioned 16 m above or northeast of the upwind perimeter of the circle. The first downwind line, DWA (Downwind Location A), was positioned such that it transected the center of the circle. The remaining two lines were positioned outside the downwind perimeter of the circle; the first, which was designated DWB (Downwind Location B), was 16 m from the circle perimeter; the second, designated DWC (Downwind Location C), ran parallel to DWB and was an additional 34 m downwind.

2.3 Task 1. Bioaerosol and Particulate Sampling Design

The sampling array was set up such that three sampling stations were positioned with equal spacing on UWA, DWB, and DWC. An additional station was placed in the center of the circle (DWA), and a mobile sampler (MOB) followed immediately behind the applicator. The MOB was considered to be a capture point for particulates and aerosols in a worse-case scenario. This sampling array design resulted in a total of 11 sampling locations, one of which was constantly mobile.

Figure 2-2 depicts the site layout in respect to bioaerosol and particulate sampling collection activities. At each of the nine stationary locations on sampling lines UWA, DWB, and DWC; two SKC BioSamplers® (hereafter referred to as biosamplers); one Andersen six-stage impactor sampler (hereafter referred to as a six-stage impactor); and two SKC Button Samplers (hereafter referred to as button samplers) were used to collect air samples over the duration of the application. The mobile unit was equipped with the same five samplers plus an additional six-stage impactor. The mobile unit was deployed such that it was beyond the wake of the applicator yet within the boundary of airflow of the suspended particles during application. The direction of the applicator relative to the position of the mobile unit was carefully planned and is discussed in the following section. The center station consisted of a GRIMM particle sampler for collecting particulate matter.

2.3.1 Sampling Station Design

2.3.1.1 Transect Stations. Although the 10 transect stations were intended to be stationary, nine of the 10 stations (all except the center station) were constructed such that they could be readily moved should a major wind shift occur during sampling, and also so that they could be quickly and easily deployed into, and removed from, the test area using minimal personnel. In order to meet these requirements, portable sampling systems were designed and built on heavy duty garden carts. Each cart was equipped with a Honda 2000EU generator to supply electrical power for the air samplers, a six-stage impactor, a Quick Take 30 pump, two biosamplers with Vac-U-Go vacuum pumps, and two button samplers with pumps.

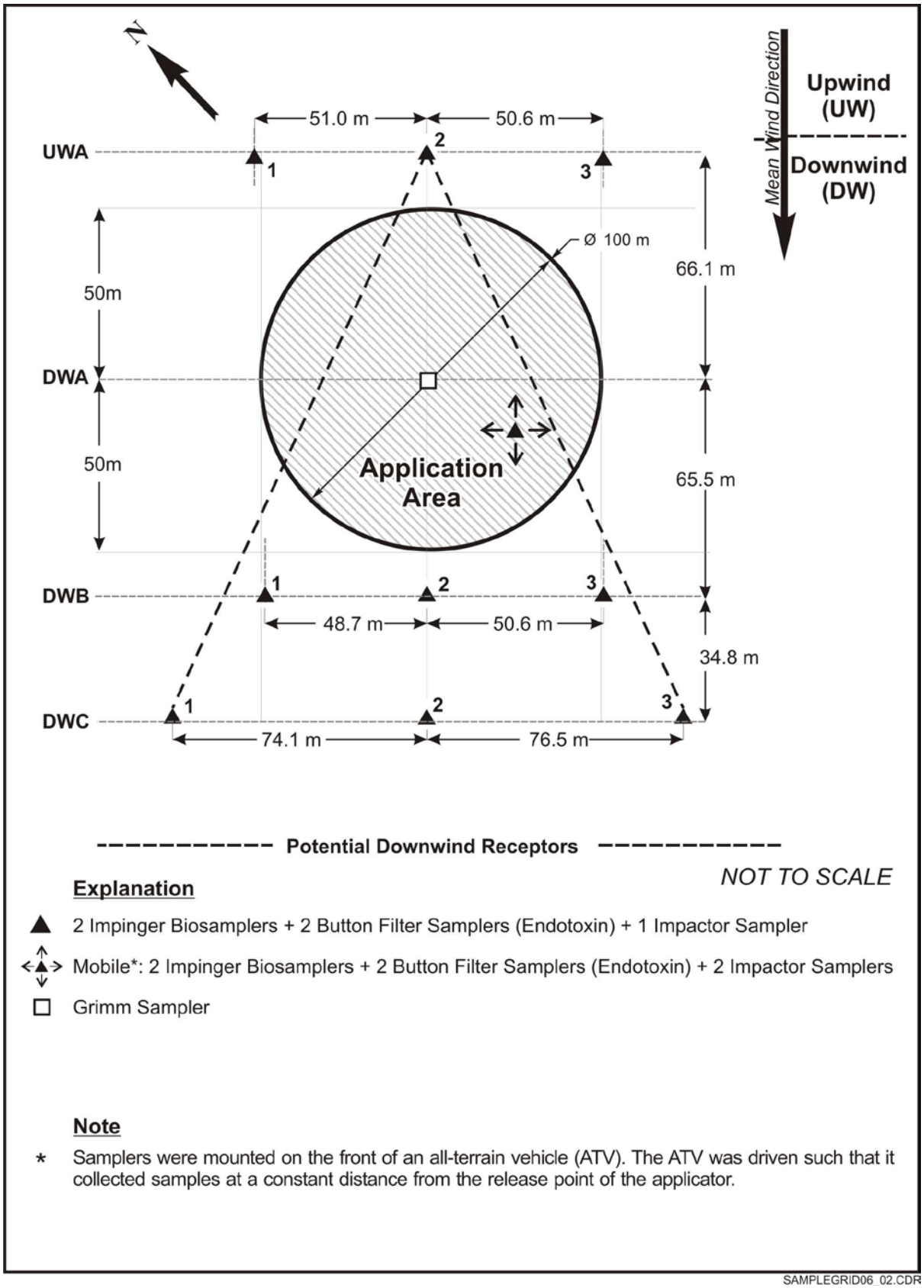


Figure 2-2. Bioaerosol and Particulate Sampling Array

Figure 2-3. Cart-Mounted Transect Sampling Equipment

Each sampling system was mounted on a tripod with a platform on which the samplers were positioned. The biosamplers were set up so that the biosampler orifice was located 1.5 m above the land surface. The six-stage impactor and duplicate button samplers were positioned at the same height on the mast. The completed unit is shown in Figure 2-3.

2.3.1.2 Mobile Unit. The mobile sampling station (Figure 2-4) consisted of a four-wheeled, all-terrain vehicle (ATV) equipped with two biosamplers, two six-stage impactors, and two button samplers with ancillary pumps and equipment. The mobile sampler was set up in the same mode as the transect samplers with the exception that it was outfitted with a second six-stage impactor.

2.3.1.3 Center Station. The center station consisted of a GRIMM sampler for collecting particulate matter. This type of sampler allows fractionation of particle size ranges.

2.4 Task 2. VOC, Ammonia, Hydrogen Sulfide, and Odor Sampling

The experimental design also included selected in-field and in-lab measurements of VOCs, ammonia, hydrogen sulfide, and various odorants. These measurements were collected in parallel with other monitoring activities being conducted for Task 1.

Figure 2-4. Mobile Unit

2.4.1 Open Path Fourier Transform Infrared Spectrometer. An OP-FTIR spectrometer was used to measure "real-time" concentrations of VOCs and ammonia in air. The system consisted of an FTIR spectrometer and a retro-reflector array. The OP-FTIR system was linked to a particle counter that summed the total amount of energy that a target compound absorbed between the FTIR and retro-reflector array. Concentrations of specific compounds were quantified using the measurement of energy absorbed within selected regions of the spectrum. Figure 2-5 shows the FTIR and retro-reflector positioning used for this research. A combination of single-path and vertical radial plume mapping (VRPM) measurements was conducted. The equipment was maintained in these positions for the duration of the sampling event described in the following sections.

2.4.2 Advective Flux Measurements. Flux chambers were used to estimate the rate and extent of volatile emissions relative to a known surface area on the ground. Figure 2-6 illustrates the flux chamber design that was used for this research. Each flux chamber was square, 120 cm on a side. One chamber was deployed in each quadrant of the circular test area with one duplicate chamber placed in one of the quadrants, for a total of five chambers. Each chamber was plumbed with a manifold assembly that was designed to capture a total of up to five discrete samples for VOC and odorant analysis.

Each chamber had an open bottom that was driven approximately 6 cm into the ground surface. The unit exhausted through an open stack that was covered loosely with aluminum foil to prevent any downdraft air from entering the inside of the chamber during sampling. Air samples were pulled into Tedlar® bags via a 12-volt miniature diaphragm pump (Gast Model # 10 D 1152-101-1052) powered by a 26-amp hour sealed battery. The pump pulled the sample from a 6.3-mm OD Teflon® tube that was inserted into the top of the flux chamber. The sample was drawn through the pump and into an Aalborg Instruments multi-tube flowmeter. The flowmeter contained a single inlet and a total of five independently controlled outlets. Each of the flowmeter outlets was plumbed with a 4.8-mm OD Teflon®

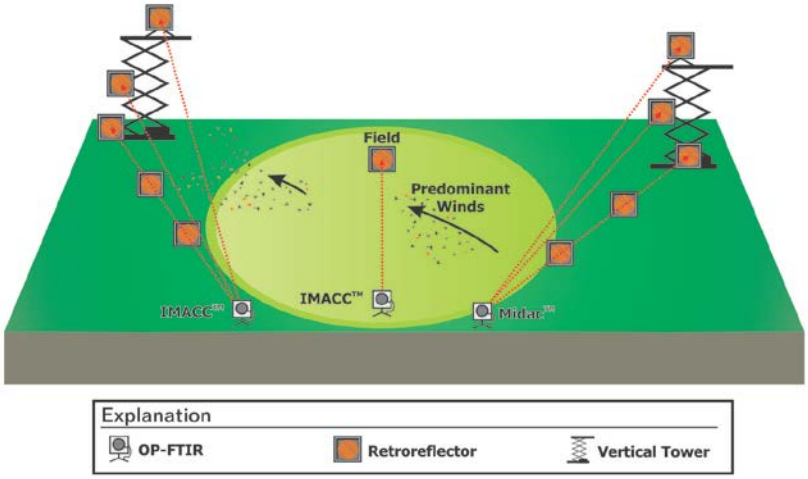


Figure 2-5. Map of the Site Layout Showing the Location of the Vertical Radial Plume Mapping Configurations and the Single-Path Measurements

tube that connected to a total of up to four Tedlar® bags with a stainless steel tube connector. The Tedlar® bags were sized larger than the target collection volumes. For each unit, three 5-L bags were used to collect samples for solid-phase microextraction (SPME) analysis and one 10-L bag was used to collect samples for odor threshold analysis (SKC Tedlar® bags, 5 L #231-05 and 12 L #231-10).

In addition, a 5-L Summa canister (under vacuum) was attached to the unit to collect samples for EPA TO15 analysis. The sample rate was controlled by attaching a fixed orifice to the inlet of the canister that allowed for the collection of a time-integrated sample.

Make-up air consisted of a pressurized cylinder of ultra-high purity (UHP) air that was metered into the base of the chamber by a rotometer. The influent and effluent flow rates were set equally to reduce the possibility of a pressure drop that would cause an influx of outside air through the stack of the chamber. The UHP air was distributed evenly inside the chamber using a circular distribution manifold that consisted of a perforated piece of Teflon® tubing. Each flux chamber was equipped with a thermocouple and HOBO datalogger so that temperature could be recorded continuously within the chamber.

2.4.3 Ammonia and Hydrogen Sulfide Measurements. In-field monitoring was conducted for ammonia and hydrogen sulfide concentrations at various locations in and around the experimental site. Ammonia and hydrogen sulfide measurements were acquired using a hand-held single sensor gas detector. These data were used to develop a qualitative "footprint" for ammonia and hydrogen sulfide about the site prior to, during, and after the application process.

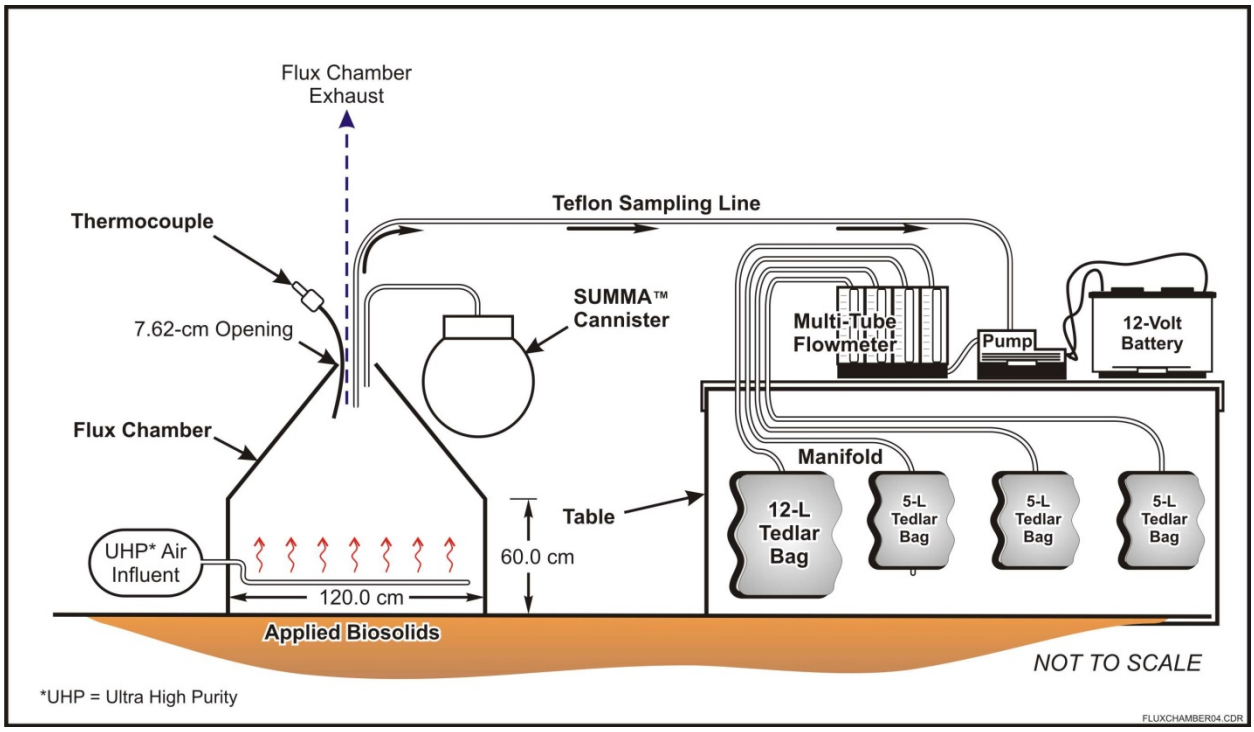


Figure 2-6. Flux Chamber Design

2.4.4 Odor Measurements. Odor, which is a primary mechanism by which the public typically becomes aware of air emissions from biosolids processing and application, was measured using two methods: in-field olfactometry (Nasal Ranger®) and in-laboratory dynamic dilution olfactometry analysis. The latter was conducted using the 12-L Tedlar®bags that collected air emissions produced by the flux chambers described above. These analyses are discussed in more detail in following sections of this report.

2.4.5 In-Laboratory Biosolids Measurements

2.4.5.1 SVOC Analysis of the Biosolids. The unapplied (stockpiled) and applied biosolids were collected and used to estimate the concentration of SVOCs in each sample. This work was conducted by Severn Trent Laboratories under contract to Battelle. Modified EPA Method SW-846 8270 was used to identify and determine the concentration of selected pesticides, polybrominated diphenyl ether (PBDE), and selected polychlorinated biphenyl (PCB) congeners.

2.4.5.2 Headspace Analysis. The headspace method of measurement was used to estimate the equilibrium concentration and type of VOCs emitted from contained grab samples that were collected from the experimental test site. Emissions in the gas space (headspace) above a containerized composite sample were measured using two analytical methods.

One method contained the sample in a specialized bottle that was equipped with a sampling side port for withdrawing the sample into a syringe for direct injection into a gas chromatograph. This sample bottle was shipped to Battelle's Atmospheric Analysis Laboratory for analysis using volatile organic analysis (VOA)-7 (a modification of EPA Method TO-15; EPA, 1999).

The second sample was containerized in a specialized Teflon® bottle and submitted to USDA laboratories, where it was analyzed for odorous chemicals using SPME and multi-dimensional gas chromatography/mass spectrometry (MD-GC-MS) procedures. These procedures and a list of analytes for both the headspace analysis approaches are described in Quality Assurance Project Plan (QAPP) #390-QC-1 (Battelle, 2004).

2.5 Task 3. Land Sampling

The land sampling component of this research (Task 3) was conducted in parallel with Task 1 and Task 2; however, the sampling regime for Task 3 extended several months beyond the day of biosolids application. The land sampling schedule and specific details regarding the sampling plan and analyses for this task are presented in the following subsections.

2.5.1 Land Sampling Field Plot Design. Unlike Tasks 1 and 2, the land sampling task was conducted in the area of the biosolids application only and not the peripheral area. The land sampling approach was developed such that three discrete plots of land were used for sampling activities. Each of the three plots was randomly sited within the 2-acre area of the biosolids test site. The plots were 3 m across by 6 m long. In the first half of the plot (3 m by 3 m), the soil was sampled at three randomly selected locations during each sampling event using a grid system. Sampling locations were not used more than one time (see Figure 2-7).

The distribution of biosolids on the day of application was measured using the second half of the plot (3 m by 3 m). This was accomplished by creating a grid for the second half of the plot and randomly selecting 20 locations within this area for sampling. Prior to biosolids application, these 20 locations were covered with a 30-cm × 30-cm square of landscaping fabric that was secured to the soil surface. Immediately after biosolids application, the squares were lifted off the soil and any biosolids on the surface of the square (along with the fabric) were placed in a sample bag. These samples were analyzed for biomass dry mass and volatile solids.

2.5.2 Land Sampling Procedures and Plan. Plant material on the soil surface and underlying soils was sampled in this study. The soil was sampled using a coring device that removed a sample measuring approximately 5 cm in diameter by 30 cm in depth. The sample handling was dependent on the analyte of interest; in some cases, samples were subdivided and, in others, samples were composited.

Information on sample handling for the land sampling component of this study is included in Section 7.0 of this report.

Land sampling included the following analytes:

- Microbial community by phospholipid fatty acid/fatty acid methyl ester (PLFA/FAME)
- Viable Helminth ova (VHO), *Salmonella*, enteric viruses, and male-specific coliphage
- Total heterotrophic bacteria (THB), fecal coliforms, *Staphylococcus aureus*, *Enterococcus* spp., *Escherichia coli*, and *Clostridium perfringens*
- Alkylphenol ethoxylates (APEs), their degradation products, and bisphenol-A (BPA)
- Soil characterization analyses (pH, percent organic matter, cation exchange capacity [CEC], percent base saturation [Ca, Mg, Na, K, and H], disturbed bulk density, USDA textural class, water-holding capacity, percent total nitrogen, phosphorous, and soluble salts)
- Biosolids dry mass/volatile solids

- Terrestrial ecotoxicity by earthworm survival, seed germination, and root elongation bioassays.

Samples of various depth ranges were collected and analyzed temporally.

2.6 Schedule of Events

Table 2-1 shows a list of sequential activities that occurred in the preparation and implementation of this study. In general, on-site activities commenced 35 days prior to the day of application of the biosolids to land in an effort to gather baseline data for the land sampling effort (Task 3). Other activities related to bioaerosol and particulate sampling and to VOC and odor sampling (Tasks 1 and 2, respectively) were conducted the day prior to application, immediately prior to application, during the application, and up to 2 days after the application. Additional post-application land sampling efforts were conducted up to 98 days after the biosolids had been land applied.

The day of application has been denoted as "Day 0". All sampling conducted prior to and after the day of application are referenced in a positive or negative fashion relative to Day 0. For example, pre-application land sampling conducted 35 days before Day 0 is referred to as Day -35.

Tables 2-2a through 2-2d present a composite list of the sampling and analyses that were conducted for this research effort, with specific detail given by day relative to the application. Each of Tables 2-2a through 2-2d are segregated by sample type (i.e., bioaerosols, VOCs, biosolids, and soil). These tables also provide relevant information about the project-specific personnel who directed the collection and/or analyses of the samples.

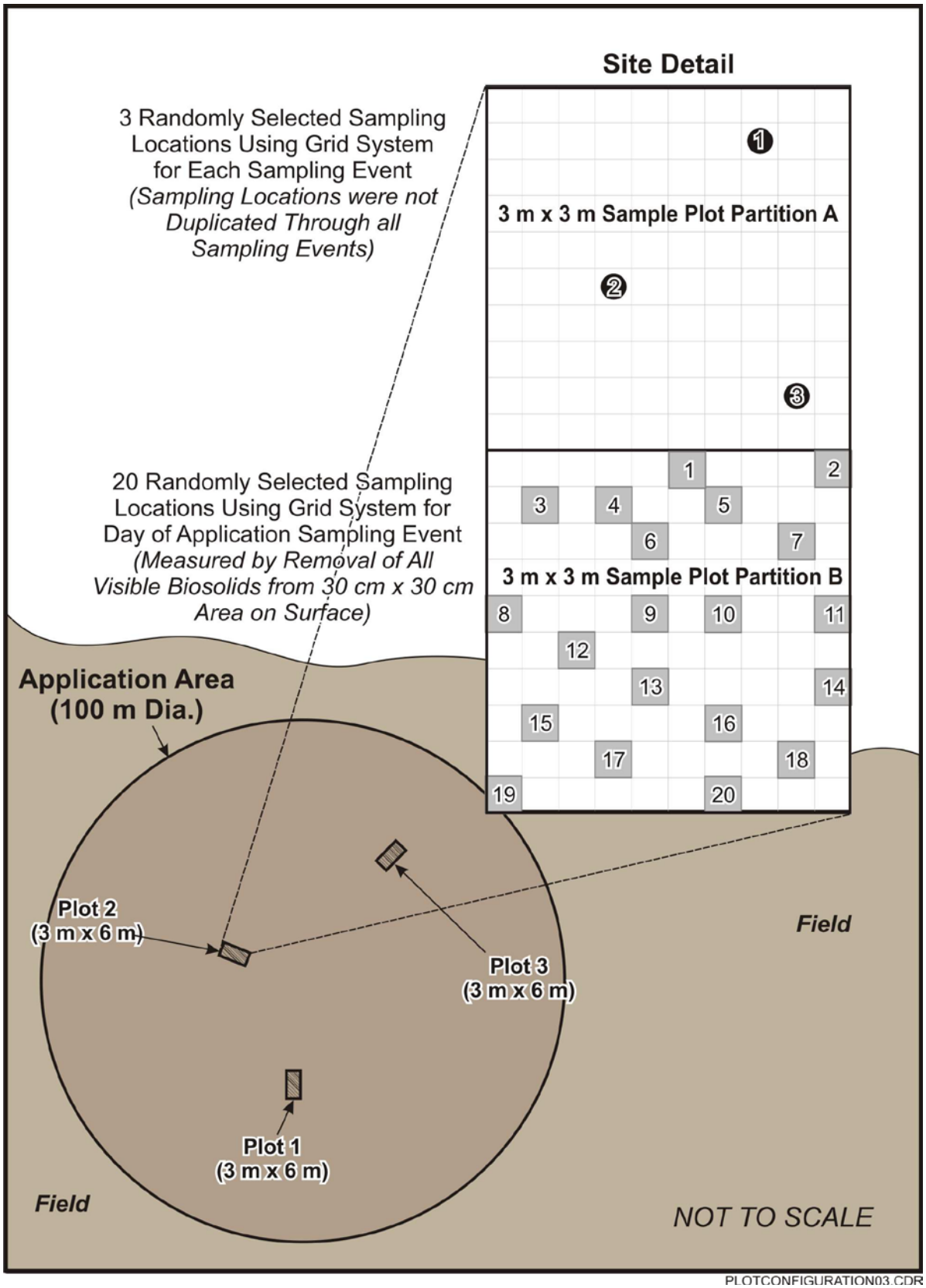


Figure 2-7. Land Sampling Plan

Table 2-1. General Schedule of Field Events

Event	Performed By	Date
Site setup, plot marking, pre application soil sampling	EPA/Battelle/ NCDA&CS	(Day -35) August 25, 2004
Initial site setup and mobilization for the application study	Battelle/ NCDA&CS	(Day -7) September 23, 2004
Conference call to discuss field schedule and logistics	EPA/USDA/Battelle and Battelle subcontractors/ NCDA&CS	(Day -5) September 25, 2004
On-site arrival of initial EPA field personnel with supplies for site preparation	EPA	(Day -4) September 26, 2004
On-site arrival of USDA bioaerosol team	USDA	(Day -4) September 26, 2004
On-site arrival of USDA VOC and odor sampling team	USDA	(Day -3) September 27, 2004
Pre-application soil sampling	EPA/Battelle/ NCDA&CS	(Day -3) September 27, 2004
Storm Delay – Morning meeting held at hotel to discuss project schedule and weather delay/Afternoon meeting held on-site to review bioaerosol sampling plan	EPA/USDA/Battelle/ Univ. of Colorado/Univ. of Arizona/CH2MHill (morning meeting only)/NCDA&CS	(Day -2) September 28, 2004
Biosolids arrived on-site at 3:30 PM and were stored in trucks underneath cover/Samples collected upon arrival/Tractor and hopper equipment arrived	NCDA&CS/USDA/EPA	(Day -2) September 28, 2004
Baseline bioaerosol and particulate sampling	USDA/EPA/NCDA&CS Battelle/Univ. of Colorado/Univ. of Arizona/ CH2MHill/Biosolids Provider	(Day -1) September 29, 2004
Biosolids application day/Flux measurements made on solids while in the truck/Biosolids removed from the trucks and dumped to form stockpile near site/Specific sampling regime is delineated in Table 4-3	USDA/EPA/NCDA&CS Battelle/Univ. of Colorado/ Univ. of Arizona/ CH2MHill/Biosolids Provider	(Day 0) September 30, 2004
Post application monitoring and field sampling (Odor monitoring and SVOC sampling-see Table 4-3)	Battelle/NCDA&CS/ CH2MHill	(Day 1-3) October 1 – 3, 2004
Post application soil sampling – See Table 4-3 and 7-1	EPA/Battelle/ NCDA&CS	(Day 14) October 14, 2004
Post application soil sampling – See Table 4-3 and 7-1	EPA/Battelle/ NCDA&CS	(Day 28) October 28, 2004
Post application soil sampling – See Table 4-3 and 7-1	EPA/Battelle/ NCDA&CS	(Day 63) December 7, 2004
Post application soil sampling – See Table 4-3 and 7-1	EPA/Battelle/ NCDA&CS	(Day 98) January 4, 2005
Site demobilization	Battelle/EPA/NCDA&CS	May 11, 2005

Table 2-2a. Bioaerosols and Particulate Sampling: Analyte, Method, Sample Frequency, and Responsible Personnel for Collection/Analyses

Analyte	Method	Responsible Party	Sample Type	Frequency
THB	USDA SOP 07.01A2	USDA	Biosamplers (split sample)	• Day -1 • Day 0, After application
Fecal coliforms	USDA SOP 7.01B2			
Escherichia coli	USDA SOP 7.01C2			
Salmonella	USDA SOP 7.02			
Staphylococcus aureus	USDA SOP 7.06			
Enterococcus spp.	USDA SOP 7.03			
Male-specific coliphage	USDA SOP 7.00 A, 7.09			
Clostridium perfringens	USDA 7.08			
Enteric viruses[(a)]	EPA, 2003 (plaque forming units [PFUs]) and EPA, 2001 (most probable number [MPN])	Battelle/Environmental Associates		
Bacterial endotoxins	USDA SOP	USDA/EPA-Cincinnati	Button Samplers	
Particulates	EPA GRIMM	EPA-Cincinnati	GRIMM Particle Sampler (in-field data collection)	• Day -1 • Day 0, After application

(a) Enumeration will reflect the possible presence of one or more of the following groups of viruses; however, counts of specific individual viruses cannot be provided for PFUs or MPN-cytopathic effect units: 24 serotypes of Coxsackie A and six serotypes of Coxsackie B, serotypes 1-34 of ECHO, Enterovirus serotypes 68-71, serotypes 1-3 of OrthoReo, and three serotypes of polio.

Table 2-2b. VOC and Odor Sampling: Analyte, Method, Sample Frequency, and Responsible Personnel for Collection/Analyses

Analyte	Method	Responsible Party	Sample Type	Frequency
OP-FTIR, EPA TO-15, and SPME	OP-FTIR	EPA-RTP	Monitored in the field	• Day -1 through Day 1
	EPA TO-15	Battelle	Flux Chambers	• Day -1 • Day 0, Arrival of biosolids at site before application • Day 0, After application (1, 3, 4, and 20 hours)
	SPME	USDA		
Ammonia and hydrogen sulfide	Hand-held monitors	Battelle/CH2MHill	Monitored in the field using hand-held instrumentation	• Day -1 through Day 2 (periodically throughout site)
Odor intensity	ASTM E544-99		Monitored in the field with olfactometers	• Day -1 through Day 2 (periodically throughout site)
Odor concentration	ASTM E679-91		Determined in the lab by an Odor Panel using samples from the flux chambers	• Day 0, After application (1, 3, 4, and 20 hours) selected flux chambers

Table 2-2c. Biosolids Sampling: Analyte, Method, Sample Frequency, and Responsible Personnel for Collection/Analyses

Analyte	Method	Responsible Party	Sample Type	Frequency
VOCs	GC/MS (VOA-7)	Battelle	Headspace Analysis of Biosolids (composite of seven randomly collected biosolid samples)	• Day 0, Arrival of biosolids at site before application • Days 1 and 2
SVOCs-Organochlorine pesticides and brominated diphenylether congeners	GC/MS (EPA Modified SW-846 8270)	Battelle/Severn Trent	Biosolids (composite of seven randomly collected biosolid samples)	• Day 0, Arrival of biosolids at site before application
SVOCs				
PLFA/FAME	EPA Standard Operating Procedure	EPA-Cincinnati	Biosolids (composite sample)	• Day 0, Arrival of biosolids at site before application
APEs/BPA	Region 5 SOP	EPA-Region 5		
Fecal coliforms	EPA 1680 (Oct., 2002) (MPN Method)	Battelle/Environmental Associates		
VHO	EPA 2003			• Day 0, Arrival of biosolids at site before application
Salmonella	EPA 1682			
Enteric viruses[(a)]	EPA, 2003 (PFUs and cell lines) and EPA, 2001 (MPN)			
Male-specific coliphage	EPA 1602	Battelle/Environmental Associates		• Day 0, Arrival of biosolids at site before application

Table 2-2c (continued). Biosolids Sampling: Analyte, Method, Sample Frequency, and Responsible Personnel for Collection/Analyses

Analyte	Method	Responsible Party	Sample Type	Frequency
THB	USDA SOP 07.01A	USDA		• Day 0, Arrival of biosolids at site before application
Fecal coliforms	USDA SOP 7.01B (Spiral Plating Method)			
Escherichia coli	USDA SOP 7.01C			
Staphylococcus aureus	USDA SOP 7.06			
Enterococcus spp.	USDA SOP 7.03			
Clostridium perfringens	USDA 7.08			
pH	SW-9045	Battelle/Agvise Laboratory		
Organic matter	Walkley Black Titrimetric			
Cation exchange capacity (CEC)	SW-9081			
Disturbed bulk density	SSSA Part 1			
% sand, silt, and clay	SSSA Part 1			
USDA textural class	SSSA Part 1			
Water holding capacity	SSSA Part 1			
% total nitrogen	SSSA Part 1			
% total phosphorous	SW-3050/6010			
% soluble salts	SSSA Part 1			
% base saturation	SSSA Part 1			
Dry mass /volatile solids	Standard Method (SM) 2540	EPA-Cincinnati	929 cm^2 Surface Sample	Immediately following application

(a) Enumeration will reflect the possible presence of one or more of the following groups of viruses; however, counts of specific individual viruses cannot be provided for PFUs or MPN-cytopathic effect units: 24 serotypes of Coxsackie A and six serotypes of Coxsackie B, serotypes 1-34 of ECHO, Enterovirus serotypes 68-71, serotypes 1-3 of OrthoReo, and three serotypes of polio.

EPA, 2003. Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge, EPA/625/R-92/013.

EPA, 2001. *Manual of Methods for Virology*, Chapter 15.

SSSA = Methods of Soil Analysis, Part 1 – Physical and Mineralogical Methods. Soil Science Society of America, Inc., Madison, WI. 1986.

Table 2-2d. Land Sampling: Analyte, Method, Sample Frequency, and Responsible Personnel for Collection/Analyses

Analyte	Method	Responsible Party	Sample Type	Frequency
PLFA/FAME	LRPCD SOP	EPA-Cincinnati	0-5, 10-15, and 20-25 cm core segments, measured on 65 mm sieved samples	• Day 35 • Day 3 • Day 0, After application • Days 14, 28, 63, and 98
APEs/BPA	EPA-Region 5 SOP	EPA-Region 5		
Fecal coliforms	EPA 1680 (Oct, 2002) (MPN Method)	Battelle/Environmental Associates	0-5 cm segment	
VHO	EPA, 2003		0-5 cm segment (composite of three samples)	• Day 35 • Day 3 • Day 0, After application • Days 28, 63, and 98
Salmonella	EPA 1682			
Enteric viruses[(a)]	EPA, 2003 (PFUs) and EPA, 2001 (MPN)			
Male-specific coliphage	EPA 1602			
THB	USDA SOP 07.01A	USDA	0-5 cm segment (composite of three samples)	• Day 35 • Day 28 • Day 98
Fecal coliforms	USDA SOP 7.01B (Spiral Plating Method)			
Escherichia coli	USDA SOP 7.01C			
Staphylococcus aureus	USDA SOP 7.06			
Enterococcus spp.	USDA SOP 7.03			
Clostridium perfringens	USDA 7.08			
Toxicity testing (earthworm mortality, seed germination, root elongation)	SOP (QAPP 33-Q3-0)	EPA-Cincinnati	3,375 cm^3 (composite of four 15 cm x 15 cm x 15 cm samples)	• Day 35 • Day 0, After application • Day 98
pH	Walkley Black Titrimetric	Battelle/Agvise Laboratory	3 samples composited each by depth (0-5, 10-15, and 20-25 cm core segments)	• Day 35 • Day 0, After application • Day 28 • Day 98
Organic Matter	SW-9081			
Cation Exchange Capacity (CEC)	SSSA Part 1			
Disturbed bulk density	SSSA Part 1			
% sand, silt, and clay	SSSA Part 1			
USDA textural class	SSSA Part 1			
Water holding capacity	SSSA Part 1			
% total nitrogen	SW-3050/6010			
% total phosphorous	SSSA Part 1			
% soluble salts	Walkley Black Titrimetric			

(a) Enumeration will reflect the possible presence of one or more of the following groups of viruses; however, counts of specific individual viruses cannot be provided for PFUs or MPN-cytopathic effect units: 24 serotypes of Coxsackie A and six serotypes of Coxsackie B, serotypes 1-34 of ECHO, Enterovirus serotypes 68-71, serotypes 1-3 of OrthoReo, and three serotypes of polio.

EPA, 2003. Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge, EPA/625/R-92/013.

EPA, 2001. *Manual of Methods for Virology*, Chapter 15.

SSSA = Methods of Soil Analysis, Part 1 – Physical and Mineralogical Methods. Soil Science Society of America, Inc., Madison, WI. 1986.

3.0 BIOSOLIDS APPLICATION

3.1 Product Selection

Anaerobic, dewatered (centrifuged) biosolids generated at a municipal wastewater treatment plant (WWTP) were specifically selected for this study. Polymer was added to the biosolids during dewatering as a normal WWTP standard practice. Consideration was given to this type of biosolids because it was desirable for the product to elicit odor and to generate particulates via surface drying, flaking, and wind erosion. At the request of the researchers, this material was pretreated with only enough lime to adjust the pH and suppress microbial growth to meet facility compliance for release (material met Class B compliance at time of release from facility). As such, this material was atypical of biosolids that would normally be released from this facility and are not produced in this manner on a regular basis. Larger doses of lime are more consistent with the normal operation of the facility.

The material once released from the WWTP was stored for approximately 1.5 days inside the truck and under cover at the NCDA&CS Piedmont Research Station prior to application. Samples were collected from the biosolids in the truck immediately after arrival and from the biosolids stockpile immediately before application, and the analytes listed in Table 2-2c were measured including fecal coliforms. Permission to hold the biosolids under cover before application was granted in the form of a permit by the State of North Carolina for the purpose of this research.

The decision to hold the biosolids prior to application was primarily driven by inclement weather (Hurricane Jeanne) that occurred during the week of scheduled application and sampling.

3.2 Application

Biosolids were applied at a rate of 10 wet tons/acre. Other than modifications to facilitate taking air and soil samples, application practices and equipment were typical of those used during normal agronomic biosolids application.

The biosolids were land applied in the test area using a Knight 8030 hopper and tractor slinger that distributed biosolids on the land surface from a discharge point on the forward left side of the hopper. Biosolids were applied at an angle with the current wind flow, so that the unit itself was not obstructing airflow to the downwind sampling array (Figure 3-1).

The applicator made one pass across the 2-acre area as depicted in Figure 3-1. When it reached the circumference of the circle, it ceased to distribute biosolids and followed along outside the circle and re-entered parallel to its first discharge point. It took 12 passes across the circle to complete the application, expending a total of 44 minutes from start to finish. Table 3-1 shows the approximate start and stop times of each pass, elapsed time per pass, the times when the hopper needed to reload, and the total expended time. The Day -1 sampling event was conducted using an empty hopper so that the particulate and fuel emissions that could potentially be produced from the movement of the equipment across the field could be captured and quantified. Careful consideration was given to ensuring that the applicator had been thoroughly cleaned in order to limit its contribution of bioaerosol emissions during the baseline sampling.

The hopper was reloaded with biosolids four times during the application. Reloading times ranged from 11 to 14 minutes measured from the point at which the hopper stopped applying biosolids to the time that it started the application again. The hopper was pulled to the stockpile location that was a

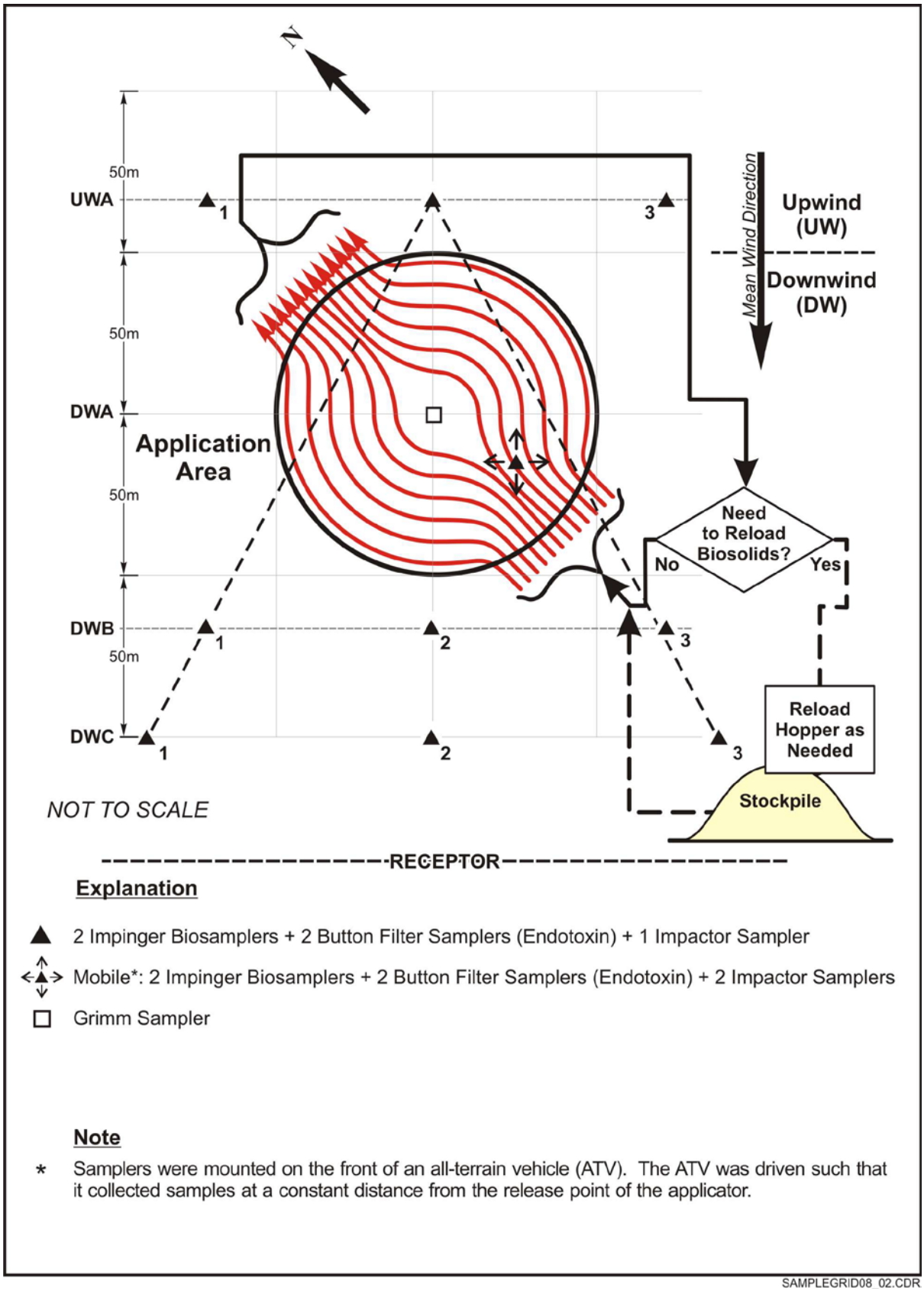


Figure 3-1. Method of Biosolids Application

Table 3-1. Application Timeline

September 29, 2004 Day -1 (Pre-application)**				September 30, 2004 Day 0 (Application)			
Start Time	**Stop Time**	**Time Elapsed (min)**	**Load Yes/No/ NA**	**Start Time**	**Stop Time**	**Time Elapsed (min)**	**Load Yes/No/ NA**
14:07	14:11	4	NA	9:37	9:42	5	No
14:20	14:25	5	NA	9:44	9:49	5	No
14:30	14:35	5	NA	10:00	10:05	5	Yes
14:39	14:41	2	NA	10:19	10:24	5	Yes
14:45	14:47	2	NA	10:25	10:29	4	No
14:52	14:55	3	NA	10:30	10:34	4	No
14:59	15:01	2	NA	10:47	10:50	3	Yes
15:07	15:10	3	NA	10:54	10:57	3	No
15:13	15:15	2	NA	10:59	11:02	3	No
15:20	15:23	3	NA	11:15	11:18	3	Yes
15:26	15:29	3	NA	11:20	11:22	2	No
15:32	15:35	3	NA	11:24	11:26	2	No
Total Elapsed Time		**37**		**Total Elapsed Time**		**44**	

NA = not applicable

** = Tractor and empty hopper were deployed to simulate application during baseline sampling.

short distance away and on the other side of the tree-line from the area of main application (see Figure 2-1). The stockpile was located at some distance away from downwind airflow to minimize the potential impacts to the in-field odor survey that was being conducted during application.

The times shown in Table 3-1, which correspond to the start and stop times of the hopper application, also correspond to the start and stop times associated with the bioaerosol sampling stations. Sampling at the nine transect upwind and downwind sampling points (three lines of three sampling points each) and on the mobile unit commenced and terminated upon the initiation and completion of each pass. This approach was designed to provide the best opportunity for capturing a detectable number of organisms and diminishing the dilution effect that would result from a continuous capture mode of sampling and prolonged waiting time during hopper reloading.

There was, however, some variance to the capture methods used during Day -1 and Day 0. During Day -1, the upwind and first line of downwind sampling units were simultaneously started and stopped in association with the application described. The second line of downwind sampling units was stopped on a 20 second delay to account for the longer travel distance of the particle plume. These samplers were started again simultaneously with the other sampling units. In practice, it turned out that this was cumbersome and difficult to accomplish simultaneously for the nine total stations. Therefore, during Day 0, this method of "staggering" sampler times was eliminated and all samplers were run for the same duration, with all the samplers running for a period deemed suitable for capturing the particle plume.

4.0 BIOSOLIDS PRODUCT RESULTS

The biosolids stockpile was analyzed for a variety of constituents (SVOCs, VOCs, indicator and pathogenic microorganisms, and physical/chemical characterization data) once it arrived on site. The reasons for these analyses varied; however, specific baseline data were needed in an effort to complete the objectives that were set for Tasks 1, 2, and 3. Therefore, biosolids as the studied matrix cross cut the three specific tasks, and the data presented here are a compilation of the data generated from these three tasks.

Upon arrival at the site, the biosolids were stored under cover at a facility approximately 1 mile away from the application area on the property of the NCDA&CS Piedmont Research Station. Once the biosolids stock arrived, it was sampled at two discrete time points (the first when the biosolids arrived on site and the second approximately 1.5 days later when the biosolids were dumped from the truck to create the stockpile).

4.1 Samples Collected from the Delivery Truck.

When the truck arrived at the site carrying the biosolids for the research study, a team of staff collected one composite grab sample constructed from seven individual grab samples collected randomly from the truck bed as specified in the QAPP.

In addition to this one composite grab sample, flux chamber measurements and sampling were conducted approximately 1.5 days after the truck arrived at the site and immediately prior to forming the biosolids stockpile on the day of application. The chamber was placed directly on top of the biosolids in the truck. During operation of the flux chamber, gas samples were collected in three 5-L Tedlar® bags and one 5-L Summa canister for SPME and TO-15 analyses, respectively.

4.1.1 Microbial Enumeration of Biosolids Grab Samples. The composite grab sample was collected and then subsampled to determine the level of *E. coli* present. The *E. coli* MPN determined from this analysis was 4.35×10^7/g-total biosolids.

4.1.2 Flux Chamber Measurement of Biosolids (Measured from the Truck). As described above, in addition to the grab samples collected from the truck, a 0.5-hr flux chamber measurement was also conducted immediately prior to removing the biosolids from the truck and creating the stockpile. This test resulted in detectable levels of dimethylsulfide and dimethyldisulfide at flux values of 3.78 and 4.54 $\mu g/m^2/hr$, respectively.

4.1.3 SPME Measurements from Samples Generated from the Flux Chambers.
The results of SPME samples collected from flux chambers positioned on biosolids in the truck are discussed in Section 6.3.2 along with the results of flux chamber SPME samples collected during biosolids application.

4.2 Samples Collected from the Biosolids Stockpile Prior to Application

On the day of application, the biosolids were dumped from the truck onto the ground to create a stockpile. Once the stockpile was formed, three composite samples (each constructed from seven individual grab samples) were collected randomly within the pile using the method mentioned previously and outlined in the QAPP. Sub-samples were removed from the composite sample for SVOC and headspace analyses using the VOA-7 method.

After these sub-samples were collected, the composite sample was contained in a sealed 5-gal bucket at ambient temperature for approximately 1 day after collection prior to being sub-sampled for the remaining suite of microbial and chemical analyses. The constituents that were analyzed from the day-old stockpile composite were:

- PLFA/FAME (Task 3)
- Fecal Coliforms (MPN Method1680 [5-Tube]) (Task 3)
- Microbial Indicators (Task 3)
  - VHO
  - *Salmonella*
  - Enteric virus
  - Male-specific coliphage
- Microbes (Task 3)
  - THB
  - Fecal coliforms
  - *S. aureus*
  - *Enterococcus spp.*
  - *E. coli*
  - *C. perfringens*
- APEs/BPA (Task 3)
- Physical/Chemical Characterization (Task 3)

4.2.1 SVOC Analysis on Biosolids. The biosolids that were collected from the composite stockpile sample were submitted for SVOC analysis using modified EPA Method SW-846 8270 for PBDE and selected pesticides and PCB congeners. Samples were also collected in the field after the application on Days 1 and 2. Three individual samples were collected from random locations within the application area. The SVOC results for these samples are presented in Table 4-1.

4.2.2 Headspace Analysis Using VOA-7. An approximate 100-g aliquot of biosolids sample was collected in triplicate and transferred into special bottles used to conduct headspace analysis. The headspace analysis was conducted according to the procedures endorsed in the QAPP by removing a headspace sample after allowing the sample to equilibrate for a period of 2 hr. The results of the analyses conducted on the biosolids from the stockpile, as well as the samples that were collected after application in the field on Day 1 and Day 2, are discussed in Section 6.3.1.

4.3 Microbial and Physical/Chemical Characterization of the Biosolids Samples Collected from the Stockpile

Tables 4-2 and 4-3 Summarize Task 3 microbial indicator and physical/chemical analyses, respectively. PLFA/FAME results for biosolids samples have been prepared such that they are relative to the land sampling effort throughout the length of the study and, therefore, are presented in Section 7.0.

Microbial indicator analyses were performed by Environmental Associates, Ltd. under contract to Battelle.

Table 4-1. SVOC Results for Biosolids Stockpile Composite Sample and Samples Collected in the Field After Application on Days 1 and 2

Analyte	9/30/2004 (Stockpile)		10/1/2004 (Day 1)		10/2/2004 (Day 2)	
	Average	Std. Dev.	Average	Std. Dev.	Average	Std. Dev.
% Moisture	78	0.6	75	0.0	74	1.5
% Solids	22	0.6	25	0.3	26	1.1
SVOCs (SW-846 8270C), µg/kg-dry wt.						
bis(2-Ethylhexyl)phthalate	16,333	3,214.6	18,000	5,000.0	15,100	5,850.6
Di-n-octyl phthalate	127	63.5	165	151.6	158	78.2
3-Methylphenol & 4-Methylphenol	35,000	7,937.3	17,000	5,567.8	5,567	3,092.5
Phenol	9,933	1,833.9	9,467	1,457.2	6,567	2,458.3
Benzo(b)fluoranthene	77	2.9	87	37.5	83	31.8
Pyrene	210	115.3	107	46.2	153	127.0
Pesticides (SW-846 8081A), µg/kg-dry wt.						
Dieldrin	13	6.9	11	8.7	13	9.4
Heptachlor	9	0.3	8	0.9	8	1.2
Methoxychlor	21	0.6	22	31.4	58	36.2

Table 4-2. Microbial Analyses of Biosolids Collected from the Stockpile Composite Sample

Date Sampled	Virus		Salmonella	H Ova	Coliphage	
	(MPN/4g)	(PFU/4g)	(MPN/4g)	(No. viable/4g)	Male-specific (PFU)	Somatic (PFU)
Day 0	<0.68	<1	>325	1.8	199	557
	2.94	<1	>325	2.5	133	616
	<0.70	<1	>325	<0.8	75	448

MPN – most probable number
PFU – plaque forming units

Additional pre-application analysis of the biosolids was performed by the USDA. The results presented as averages of triplicate biosolids samples collected from the pile immediately prior to application are as follows:

- THB: 1.6×10^{11} CFU/gdw total solids
- Total Coliforms*:* 1.4×10^{9} CFU/gdw total solids (consisted of only fecal coliforms)
- Fecal Coliforms: 2.3×10^{9} CFU/gdw total solids
- *Enterococcus* spp.: 8.2×10^{5} CFU/gdw total solids
- *Staphylococcus aureus*: none detected
- *Salmonella*: 6.33 MPN/gdw total solids (confirmed *Salmonella enteritidis* in all three replicates)

Physical/chemical analyses were conducted by Agvise Laboratories under contract to Battelle. Table 4-3 summarizes these data for the biosolids that were collected from the stockpile composite sample.

Table 4-3. Physical/Chemical Constituents Results for Biosolids Collected from the Stockpile Composite Sample

Parameter	Value
Sand (%)	5
Silt (%)	78.6
Clay (%)	16.4
USDA Textural class	Silt Loam
Bulk Density (gm/cc)	0.89
Cation Exchange Capacity (meq/100g)	22
Moisture @1/3 bar (%)	158.4 (supersaturated)
Moisture @15 bar (%)	75.6
pH (H_2O)	7.4
Total P (mg/kg)	24,453
Olsen Phosphorus (mg/kg)	176
Soluble Salts (mmhos/cm)	4.09
Base Saturation Data (mg/kg)	
Ca	823
Mg	2,231
Na	627
K	221
H	26

5.0 TASK 1. BIOAEROSOL AND PARTICULATE SAMPLING RESULTS

5.1 Objectives

In Task 1, aerosol emissions from biosolids were sampled to evaluate methods that measure the concentrations of selected bacteria, fungi, viruses, bacterial endotoxins, and particulates. These aerosol emissions were sampled prior to, during, and after biosolids application. Samplers were located upwind, within, and downwind of the application area. The primary objectives for bioaerosol and particulate sampling were to:

1. Characterize the type and concentration of the suite of viable bioaerosol components selected for analysis (seven bacteria, culturable enteric viruses, and male-specific coliphage) as well as particulates ($\leq$ 5.0 μm)
2. Determine if the bioaerosol components were emitted and transported to several points downwind of the biosolids application area under the circumstances investigated
3. Investigate the collection performance of the six-stage impactors, biosamplers, and GRIMM sampler as applied in this field study setting.

Table 2.2a lists the analytes, methods, sample types, and sample frequency for the bioaerosol and particulate sampling efforts. Analytical methods were evaluated based on whether data acceptance criteria specified in the QAPP were achieved.

5.2 Bioaerosol and Particulate Matter Sampling

A limited number of quantitative field studies that involved bioaerosols associated with biosolids management activities have been performed (Tanner, *et al*., 2008; Low *et al*., 2007; Paez-Rubio, *et a*l., 2007; Baertsch, *et al*., 2007; Brooks *et al*., 2005; Brooks *et al*., 2004; Tanner, *et al*., 2005; Rusin, *et al*., 2003; NIOSH, 1998; and Pillai, *et al*., 1996). Some of these studies have shown downwind concentrations of heterotrophic organisms at biosolids application sites to be greater than upwind concentrations, but these levels were not considered to pose a public health concern because indicator organisms (such as coliforms) were not detected at any significant distance from the biosolids source.

Bioaerosols were defined for this study as aerosolized particles of biological origin or activity that may affect living things through infectivity, allergenicity, toxicity, pharmacological impacts, or other processes. Particle sizes may range from aerodynamic diameters of ca. 0.5 to 100 μm (Hirst, 1995). In effect, bioaerosols have physical, biological, and chemical attributes and may contain fragments or parts of the original intact organisms. For this research, a particle size analyzer (GRIMM) located in the center of the application area (Figure 2-2) measured particles in the size range of 0.23 to 20 μm. Specific particle sizes captured by the bioaerosol sampling equipment (impactors and biosamplers) are unknown but assumed to be within this size range.

Enteric viruses were assayed from air samples. The procedures used to conduct the assay required that the virus be active and capable of infecting the Buffalo Green Monkey Kidney cell line. Many enteric viruses that potentially could be present in the biosolids are not known to infect this cell line, and others will not infect any cell line at all or only sporadically. Thus, the active virus populations that were assayed reflected the possible presence of one or more of the following groups of viruses (plaque-forming units [PFUs] or most probable number [MPN]-cytopathic effect units; 24 serotypes of Coxsackie A and six serotypes of Coxsackie B; serotypes 1-34 of ECHO; Enterovirus serotypes 68-71; serotypes 1,2,3 OrthoReo; and three serotypes of polio). Counts of specific individual viruses could not

be provided because of limitations of the methods, and polymerase chain reaction (PCR) analyses were not conducted for this work.

Coliphages are viruses that infect certain coliform bacteria, but not humans, animals, or plants. Those investigated in this study infect *E. coli*. Methods of detection and enumeration can be conducted in most microbiology laboratories using plaque assays. In contrast, culture-based methods of detection and enumeration of enteric viruses require specialized facilities and training in tissue culture, a feature not available in all microbiology labs. Coliphage analysis is significantly less expensive than analysis for enteric viruses. Because of these features, coliphages have served as surrogates for enteric viruses and they were included for assaying in this study.

Fecal coliforms were included as a reference standard method. The pre-eminent fecal coliform, *E.coli*, historically has served as an indicator of fecal pollution. It has the good characteristics of a fecal indicator, such as not normally being pathogenic to humans, and is present at concentrations much higher than the pathogens it predicts (Scott *et al*., 2002). Methods for their detection in air are not standardized; standard methods for their detection in water have been adapted for analyzing airborne organisms collected using liquid impingement air sampling in these studies.

Salmonella spp. are fecal pathogens and are thus a public health concern. Methods for their detection in biosolids are standardized (EPA Method 1682). Methods for their detection in air are not standardized; standard methods for their detection in water were adapted for the analysis of airborne organisms collected using liquid impingement air sampling in these studies.

Enterococcus spp. is a renamed subgroup of fecal streptococci; there are at least five species, *Enterococcus faecalis, E. faecium, E. durans, E. gallinarum,* and *E. avium,* that are differentiated from other streptococci by their ability to grow in the presence of stressors, specifically 6.5% NaCl, pH (9.6) and 45°C. Two species, *E. faecalis* and *E. faecium,* are most frequently found in humans. *Enterococcus* spp. have been used successfully as indicators of fecal pollution and are especially reliable as indicators of health risk in marine and recreational waters. However, environmental reservoirs of *Enterococcus* spp. exist, and they may re-grow when released into the environment (Scott *et al*., 2002).

In contrast to the coliforms *E. coli* and *Salmonella* spp., which are gram-negative bacteria, *Enterococcus* spp., *Clostridium perfringens,* and *Staphylococcus aureus* are gram-positive bacteria. *C. perfringens* is the only bacterium in the specific assay suite that is a strict anaerobe and endospore former. They were selected for inclusion in this study because: 1) *Enterococcus* spp. are also indicators of fecal contamination, 2) they are used in water quality evaluations, 3) *S. aureus* has been implicated as the causal agent in human infections speculated to have resulted from direct contact with land applied biosolids (EPA, 1992), and 4) *C. perfringens* is regarded by some researchers as a useful, very conservative indicator of fecal bacterial contamination. Methods for their detection in air are not standardized; standard methods for their detection in water were adapted for the analysis of airborne organisms collected using liquid impingement air sampling in these studies.

THB were included as a positive control of sampler operation with regard to viable bacteria in ambient air. Heterotrophic bacteria include the saprophytic aerobes and facultative anaerobes that are naturally present in soil, on plant surfaces, in air, and in water. They include many beneficial, non-pathogenic bacteria that degrade organic matter. Their concentrations in soil, water, and air depend on vegetation type and amount, local climate and soil conditions, animal and human activities, and circumstances in the general vicinity in which the soil, water, or air sample is obtained. In this study, their presence in air samples upwind and downwind demonstrated that the air samplers were operating sufficiently well to collect viable microbes and permitted characterization of concentration variations across the sample grid. Methods for their detection in air are not standardized; standard methods for their

detection in water were adapted for the analysis of airborne organisms collected using liquid impingement air sampling in these studies.

Sampling for endotoxin (lipopolysaccharides containing Lipid A from all gram-negative bacteria) was performed on air samples. When inhaled and respired, endotoxin can elicit a variety of well-characterized responses in mucous membranes (eyes, nose, sinus, throat, bronchi, and lungs) and systemic complaints (fever, malaise, and pulmonary function distress), some of which are among those commonly described by persons reporting illness they associate with biosolids application near their residences.

Sampling and testing for airborne fungi were conducted to assess "mold" in air. Several protocols for sampling are available. The sampling and culturing methods used in this study were designed to provide an estimate of total viable fungal particles.

5.3 Overview of Field Operations

A total of nine bioaerosol sampling stations and one mobile bioaerosol sampling unit were deployed for testing and were located within and outside a 100-m diameter (circular) study area described in Section 2.3 and shown in Figure 2-2. The GRIMM optical scanning counter was placed in the center of the application field.

One upwind (UWA) and two downwind (DWB and DWC) parallel sampling transects were located outside the 100-m circular test area (see Figure 2-2). Each transect contained a total of three sampling stations that were spaced laterally to increase the collection area of the sampling field, as opposed to clustering the samplers within narrow zones or regions. The sampling station layout for transects UWA, DWB, and DWC included three upwind stations (UWA- the center station located 16 m upwind from the top boundary of the application area), three stations in the first downwind transect (DWB- the center station located 16 m downwind of the lower boundary of the application area), and three stations in the second downwind transect (DWC- the center station located 50 m downwind of the lower boundary of the application area). The stations for transects UWA and DWB were 75 m apart within each transect. The stations for transect DWC were approximately 90 m apart.

The field design allowed for the physical movement of each station because the samplers and their ancillary equipment (pumps and power sources) were secured on pull carts so they could be re-positioned readily if necessitated by varying wind patterns. Each of the sampling stations contained two biosamplers, one six-stage impactor, and two button samplers. The cart-mounted bioaerosol sampling equipment was shown previously in Figure 2-3.

A MOB was also deployed in the application area. The MOB consisted of an ATV with two biosamplers, two six-stage impactors, and two button samplers affixed to a modified plate on the forward side of the vehicle. The ATV followed a path approximately 8-10 m to the rear and downwind of the Knight 8030 side-discharging hopper that applied the biosolids. When the hopper moved off site to reload, the MOB waited inside the application area and commenced sampling again once biosolids application was re-initiated. The MOB is shown sampling behind the side-discharging applicator in Figure 5-1.

5.3.1 Application Schedule. Due to the logistics of testing and the amount of labor necessary for on-site sample processing and analysis, the control trial and the biosolids application test were conducted on separate consecutive days. The control trial was conducted first and involved all of the same activities that occurred during the biosolids application, including the movement of the biosolids application machinery, except that during the control trial the discharge hopper was not loaded with biosolids and was cleaned by pressure spraying prior to use. Therefore, biological or particulate matter contribution in

Figure 5-1. MOB Conducting Bioaerosol Sampling Approximately 8-10 m Behind Biosolids Applicator

aerosols due to dust generation during movement of the application equipment could be determined separately from that which was generated from biosolids.

5.3.2 Operational Schedule. For both the control trial and the actual biosolids application, the aerosol samplers were operated intermittently, with sampling focused during the actual time that the applicator moved through the application area and with a reasonable allowance time for particulate transport (2 minutes) to downwind stations. When the applicator moved out of the application area, sampling was disengaged in an effort to reduce overload on the impactor agar plates and evaporative loss of biosampler fluid associated with longer collection periods (Barth, 2007).

The biosolids applicator moved across the field as shown in Figure 3-1 and moved off site to the stockpile intermittently when it needed to reload. A total of 44 minutes were required to complete the application. In a similar manner for the MOB, the biosamplers, six-stage impactor, and button samplers were operated during the period when the applicator was traveling across the application area. The impactor agar plates were replaced once during the application to reduce overloading on the agar plates.

5.3.3 Aerosol Sample Collection. The biosamplers were operated at an airflow rate of 12.5 L/min. The collection fluid consisted of 20 mL of a sterile 0.02-M phosphate buffered (pH 7.4) solution (PBS) that was subsequently transferred to R2A agar plates following APHA Method 9215 (APHA, 1992), with subsequent analysis for bacteria (only). The biosamplers were foil covered to block ultraviolet light, and periodic addition of the PBS was implemented to replace evaporated fluid.

The six-stage impactors were operated at an airflow rate of 28.7 L/min and contained two-section agar plates that were separately analyzed for viable bacteria and fungi (with subsequent adjustment for the positive control corrections). The fungi section of the agar plate contained Oxgall media (Difco) supplemented with 50 mg/L of vancomycin and streptomycin to inhibit bacterial growth. The bacteria section of the agar plate contained R2A agar (Difco), supplemented with 0.5% pyruvate to assist the resuscitation of stressed bacteria.

Particulate size and mass were monitored immediately before and during both the control trial and the biosolids application. The GRIMM Model 1108 optical scanning counter was operated at an airflow rate of 1.2 L/min.

5.3.3.1 Biosampler. The biosamplers were secured on their tripod masts at approximately 1.5 m above the ground surface and inside the carts such that the orifices were oriented into the wind flow. When sampling commenced, the Parafilm™ cover was removed from the orifices of the biosamplers, the biosampler outlets were connected to a designated pump, and the pumps were turned on. Figure 5-2 shows the biosamplers being operated in the field during application testing.

Figure 5-2. Downwind B (DWB) Biosampler and Operator

When the sampling event was completed, each biosampler was disconnected from its pump and immediately taken to the on-site mobile lab for sample processing and analysis. The liquid sample was aseptically transferred into a sterile sample container for analysis. The sample volume was fractioned as indicated in Figure 5-3 for the individual analyses already discussed in Section 5.2.

On-site processing was conducted in duplicate for each bioaerosol liquid sample. A 5.0-mL aliquot was aseptically transferred into a separate screw-cap centrifuge tube and frozen for off-site analysis of enteric viruses. Another 3.0 mL was used to conduct the *Salmonella* spp. and *E. coli* pathogen assays. The remaining sample was distributed and analyzed for *C. perfringens* (1.0 mL), male-specific coliphage (1.0 mL), *Salmonella* (MPN) for any samples that tested positive for the pathogen assay (4 mL), THBs (1.0 mL), fecal coliforms (1.0 mL), *E. coli* (1.0 mL), *Enterococcus* (1.0 mL), *S. aureus* (1.0 mL), and bacterial bioburden (1.0 mL). A 4.5-mL aliquot was transferred and stored in reserve at 4°C.

5.3.3.2 Six-Stage Impactor. When sampling was completed, each six-stage impactor was disconnected from its pump and taken to the on-site mobile lab. Agar plates from the six-stage impactor were aseptically removed and recovered with sterile lids, labeled, and incubated at 37°C. At 24 and 48 hr, the

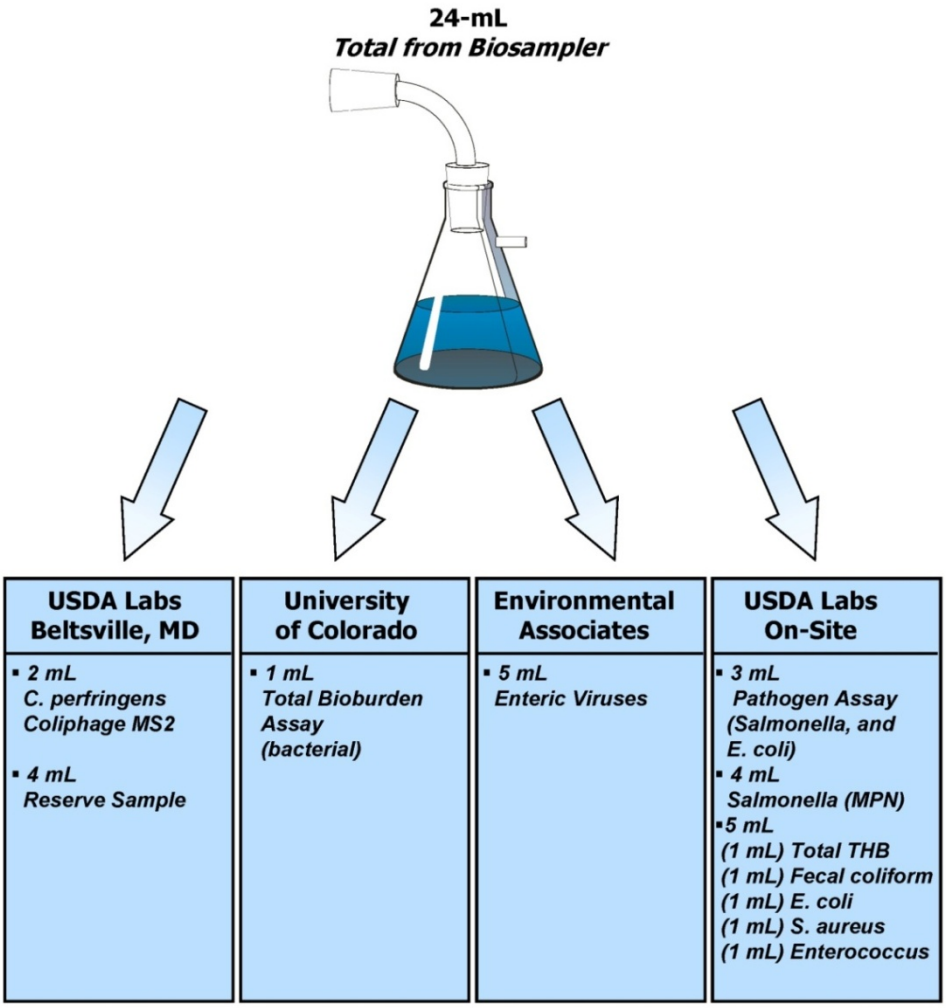


Figure 5-3. Distribution of Biosampler Fluid for Bacterial and Viral Analyses

plates were examined for colony growth and the number of colonies corresponding to the stage sieve hole pattern were counted. The positive hole correction table (Macher, 1989) was used to convert the count to a maximum likelihood number of positives to account for the possibility of multiple impactions.

5.3.3.3 Button Sampler. The button samplers used to obtain bacterial endotoxin samples were operated in parallel with the biosamplers and six-stage impactors. After sampling was completed, the sample filters were harvested and stored at 4°C in the mobile laboratory until they were packaged for overnight shipment to the laboratory for extraction and endotoxin analysis. Unfortunately, problems were encountered in the field with the filters in many of the filter units, resulting in wrinkling and tearing of the filters and ultimately negatively impacting their performance. Additional complications were encountered in the laboratory during further processing and extraction. Due to these complications, the validity of these data was questioned and, therefore, are not reported. There is no further discussion of endotoxin in this report.

5.3.3.4 GRIMM Particle Analyzer. A GRIMM particle analyzer/dust monitor Model 1.108 was used for the continuous measurement of particles in the air. The GRIMM monitored single-particle

counts using a light-scattering technology and recorded the particle data on a data storage card. After completion of the test, the card was removed and the data were downloaded.

5.4 Bioaerosol and Particulate Matter Results

The prevailing wind direction for this site is nominally from the north or northeast at this time of the year according to historical records. Based on this information, the sample collection array was designed with upwind samplers to the northeast and downwind samplers to the southwest of the application area (see Figure 2-2). However, during the control trial (Day -1), the ground-level wind direction was unexpectedly from the southwest (Figure 5-4) with an average magnitude of 1.1 m/s and minimal gusting. As a result, the design as depicted in Figure 2-2 acted in reverse on this day with the single upwind transect serving as the downwind sampling stations, and the double downwind transects serving as the upwind sampling stations. Although this direction was not as expected, the control trial results are still considered valid since no statistical differences in the upwind (acting as the downwind) and two downwind (acting as the upwind) stationary zones were observed, as would be anticipated with no biosolids being applied.

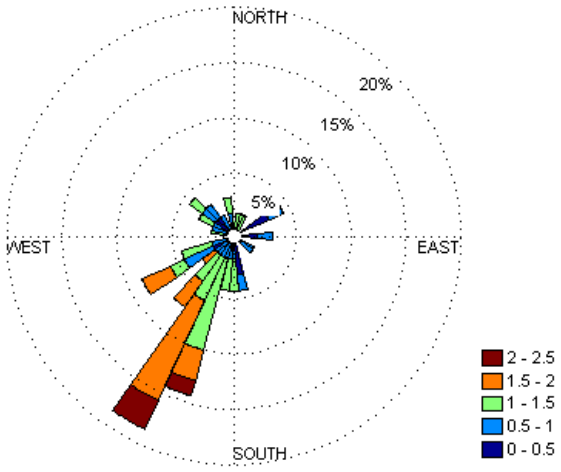
During biosolids application (Day 0), the wind direction was predominantly from the north and the design depicted in Figure 2-2 functioned as expected. On this day, wind was very light with an average magnitude of 0.6 m/s.

5.4.1 Bioaerosol Results and Analysis. Fecal coliforms, *E.coli*, *Salmonella* spp., *S. aureus*, *C. perfringens*, *Enterococcus spp.*, and coliphage were not detected in any of the bioaerosol samples collected anywhere on the site either in the control trial or during biosolids application. This also was the case for the MOB that sampled within 8-10 m of and directly behind the discharged biosolids.

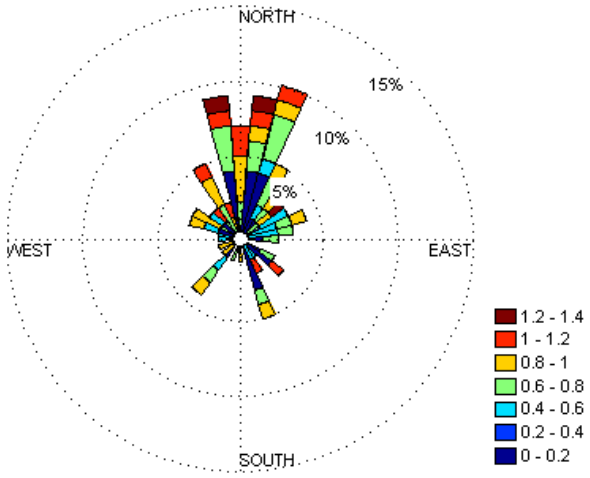
Enteric virus analyses were conducted using the PFU and MPN procedures. Virus analyses were only performed on the mid-line sample stations (see Figure 2-2; UWA-2, MOB, DWB-2, and DWC-2) for the control trial and application test, resulting in a total of 16 samples for virus analysis. Four additional blank samples were included for quality control purposes. The QAPP specified that PCR would be performed on these 16 samples only if they were positive for PFU and MPN. The remaining 32 samples (from the remaining stations) were frozen and were to be analyzed for PFU/MPN and possibly PCR only after it had been determined that the first 16 samples yielded positive results. There were no positive results for enteric virus in the PFU and MPN analyses that were conducted for the initial 16 samples; therefore, no further analyses were conducted on these samples or on the remaining 32 samples that were kept frozen.

THB were assayed and detected in all bioaerosol samples collected with the biosamplers for both the control trial and biosolids application test. THB were also detected on agar plates in the six-stage impactors. Their presence in both upwind and downwind air samples demonstrated that the air samplers were operating sufficiently well to collect viable microbes. Total fungi were also assayed and detected in the six-stage impactors.

The bioaerosol data for each of the three stations within a given transect (UWA, DWB, and DWC) were averaged (e.g., the THB results for UWA stations 1, 2, and 3 were averaged, representing the entire upwind zone). The data were analyzed to: 1) determine if there were significant differences in THB and fungi counts between the control trial and the biosolids application test, and 2) determine if there were significant differences in the THB and total fungi counts between upwind and downwind locations.



(A) Day -1 (Control Trial - September 29, 2004 from 2:00 - 3:30 PM)



(B) Day 0 (Application Test - September 30, 2004 from 9:30 – 11:30 AM)

Figure 5-4. Predominant Wind Directions and Velocities (m/s) During Biosolids Control Trial and Application Test

Descriptive and inferential statistical comparisons were performed. The specific statistical methods used depended on whether the data distribution was normal or log-normal after log transformation. The geometric mean and geometric standard deviation were used in statistical comparisons when the data were transformed. All statistical analyses were performed using Statistical Analysis Software (SAS) MEANS, TTEST, and MIXED procedures (SAS, 2003). Scheffe adjustments were made for multiple comparisons, and *t*-tests were used for single comparisons. Probabilities < 0.05 were considered statistically significant.

The airborne concentrations (CFU/m^3) of bacteria and fungi collected with the six-stage impactors and the concentrations of bacteria (only) collected with the biosamplers for both the control trial and the application test are presented in Figure 5-5. Biosampler data for bacteria for each sampling transect tended to be more variable than were the six-stage impactor data, particularly for transect UWA during the control trial and for MOB and transect DWC during the application test, even though more biosamplers were used for each transect. Since the six-stage impactor is the traditional sampling device used in bioaerosol sampling, no inferential statistics were attempted using the biosampler data. The samples on which the enteric virus analyses were conducted were collected by the biosamplers.

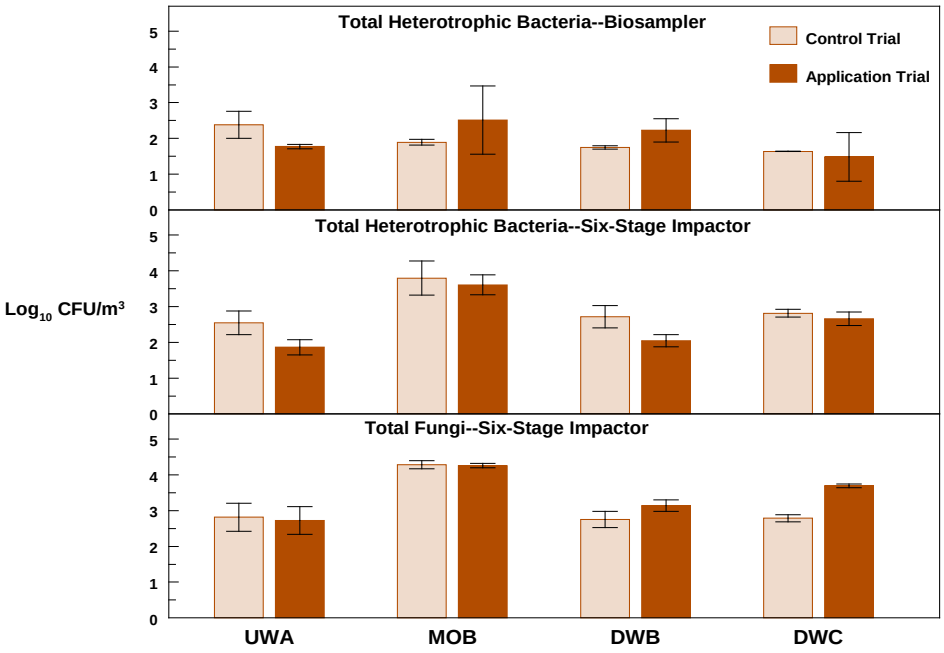


Figure 5-5. Bioaerosol Concentrations of Microorganisms for Mobile, Upwind, and Downwind Sampling Locations

Inferential statistics were applied to the six-stage impactor data to determine which sampling locations exhibited statistically significantly different concentrations of THB and total fungi. Table 5-1 summarizes those comparisons of sampling locations during the control trial and the application test that were found to have statistically significant differences (defined as $p < 0.05$). Note that the sampling locations in the right-hand column are more downwind than the sampling locations in the left-hand column to which they are compared.

Table 5-1. Summary of Sampling Location Comparisons with Statistically Significant Differences for THB and Total Fungi (Six-Stage Impactor Data Only)

Microorganism	Sampling Period	Zone Comparison	Probability
THB	Control Trial	UWA vs. MOB	<0.02
		MOB vs. DWB	<0.04
	Application Test	UWA vs. MOB	<0.01
		UWA vs. DWC	<0.01
		MOB vs. DWB	<0.01
		MOB vs. DWC	<0.02
		DWB vs. DWC	<0.04
Total Fungi	Control Trial	UWA vs. MOB	<0.01
		MOB vs. DWB	<0.02
		MOB vs. DWC	<0.02
	Application Test	UWA vs. MOB	<0.01
		UWA vs. DWC	<0.02
		MOB vs. DWB	<0.04

The mean airborne concentrations of THB collected with the six-stage impactors were greater during the control trial than during the application test for the MOB and all stationary sampling locations. As shown in Figure 5-5, similar concentration profiles were observed for the various sampling locations during both the control trial and application test. During both sampling periods, statistically significant differences were observed between UWA vs. MOB and between MOB vs. DWB. The fact that the MOB sampler exhibited differences from two of the three upwind and downwind sampling transects for the six-stage impactors during the control trial suggests that the application machinery (without biosolids) aerosolized dust particles containing microorganisms; therefore, any differences among these locations during the application trial may or may not be attributable to the biosolids source. During the application test, statistically significant differences were also noted for UWA vs. DWC and MOB vs. DWC. A statistically significant difference was also noted for DWB vs. DWC, where the mean THB concentrations for DWC were greater than those for DWB. This was an unexpected observation as it would be anticipated that the concentrations would decrease with distance from the application area due to factors such as dispersion.

The mean airborne concentrations of fungi were slightly greater during the control trial than during the application test for UWA and MOB. In contrast to the THB data, the mean concentrations during the control trial were less for DWB and DWC than during the application test. As shown in Figure 5-5, consistent with the THB data, similar concentration profiles were observed over the various sampling locations for the control trial and application test. Also consistent with the THB data, statistically significant differences were observed during both sampling periods between UWA vs. MOB and between MOB vs. DWB. Again, any differences noted among these locations during the application test may or may not be attributable to the biosolids source. The concentration differences for MOB vs. DWC were statistically significant for the control trial but not for the application test. During the application test, statistically significant differences were also observed for UWA vs. DWC. Contrary to the THB data, DWB was not statistically different from DWC during the application test as the standard error of the mean zonal difference (log-scale) of the bacteria was smaller than for the fungi.

The differences in bioaerosol concentrations for both types of microorganisms (THB and total fungi) during the two sampling periods may have been influenced by documented differences in

environmental conditions such as the time of day of sampling (2:00 – 3:30 PM for the control trial vs. 9:30 – 11:30 AM the next day for the application test), ambient air temperature (25°C vs. 19.8°C, respectively), relative humidity (50.2% vs. 86.0 %, respectively), and solar index (644 watts/m^2 vs. 404 watts/m^2, respectively). The effects of these variables or combinations thereof were not evaluated. The differences in these environmental conditions could be partially mitigated by sampling at the same time on consecutive days.

The accuracy and precision of the methods used for collecting microorganisms in this field setting were not determined. Even with expected high collection efficiencies of airborne bacteria, laboratory assays for individual microorganisms may indicate low recoveries. Microbial ecology studies have shown the culturability of microorganisms is low compared to actual counts in many environmental settings (Fabbian *et al*., 2004). For example, the recovery efficiency for *S. aureus* seeded in a biosolids sample was 8.7% (Rusin *et al*., 2003). In a bioaerosol study, less than 10% of the aerosolized bacteria were capable of forming visible colonies with culture techniques (Heidelberg *et al*., 1997).

5.4.2 Particulate Matter. The mean values for the total mass concentration (µg/m^3) of all particulates ≤5.0 µm (in the general inhalable size range for many bacteria and fungi) detected per unit time by the GRIMM sampler immediately before the control trial was initiated, during the control trial, immediately before the application test was initiated, and during the application test are presented in Figure 5-6. The control sampling period produced greater mass capture than did the pre-control sampling period after log-transformation and Satterwaite adjustment of the *t*-test due to unequal variances. The increase in suspended particulate mass captured (approximately 90 µg/m^3/min) was likely due to equipment activity on the application field. No statistically significant differences in particulate mass captured were noted between the pre-application trial period and the application sampling period. One reason a difference in

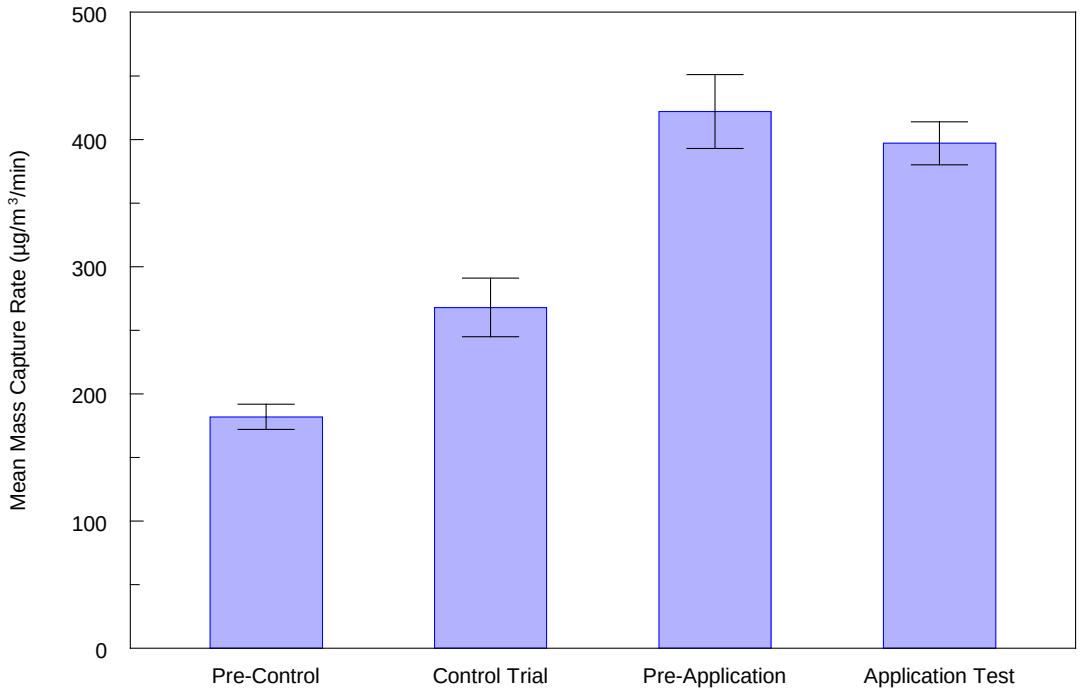


Figure 5-6. Mean Mass of Airborne Particulates (≤ 5.0 µm) Captured by the GRIMM Sampler Immediately Before and During Control and Application Sampling Periods

mass captured was not observed between these two periods may have been due to the biosolids used in this study, which appeared to have reduced friability. The adsorption of microorganisms to particulates in biosolids is one of the factors thought historically to influence the amount of bioaerosolized microorganisms collected during field studies (Tanner, 2005).

5.5 Conclusions

In this specific outdoor environment, differences in bacterial and fungal counts were observed for the six-stage impactor data for the various sampling locations during both the control trial and application test. There were expected concentration differences at the MOB sampling location, but unexpected increases were noted in the mean concentrations for THB at DWC compared to DWB during the application test. Fine particulates of microorganism size did not appear to be aerosolized during biosolids application. This lack of observed aerosolization may have been influenced by environmental conditions and the biosolids additives, primarily polymer, and processing operations. The biosolids tended to have a sticky or more gel-like consistency that may have diminished their friability and ability to produce fine aerosolized particles.

5.6 Discussion and Lessons Learned

The nature of the biosolids used in this research may have had an impact on the bioaerosol data and other data sets in this study. The sludge processing at the municipal WWTP produced a biosolids material that was sticky and gelatinous, substantially reducing its friability and perhaps limiting its ability to be dispersed as fine particles into the air. These properties may, in turn, have negatively impacted the capture and detection of aerosolized microorganisms. For future studies in which the primary objective is to maximize dispersion of biosolids and associated chemicals and microorganisms, it is recommended that a more friable biosolids matrix be selected that maximizes uniform distribution of fine, flaky particles to the soil and into the air. Application of liquid biosolids may also yield a more uniform distribution of droplets to the soil and into the air. Conversely, application of a more agglomerating biosolids product, from a practical sense, may help restrict the applied material to the immediate area and limit the spread of airborne particles to downwind receptors.

The bioaerosol conclusions from this study may have been affected by the physical site location and weather conditions. It is plausible that land application of biosolids during a humid, cloudy day might allow air-sensitive and UV-sensitive bacteria to survive longer. In future studies involving bioaerosols, a flat site that minimizes elevation differences in collection devices is recommended. Furthermore, sites with consistent wind velocities and directions will simplify data interpretation. Finally, it would be helpful if the selected sites were not surrounded by heavy vegetation to minimize external microorganism influences form grasses, plants, and trees.

Since it was of interest (particularly for Task 3) to conduct this work at a site where biosolids had never been land applied, there was no opportunity within the study design to replicate the application tests. It is recommended that for future work a study design with focus on bioaerosol monitoring be considered so that tests can be replicated to increase the power of the study and reduce uncertainties.

In addition, it would be appropriate to consider the use of air dispersion models to estimate airflow regimes and guide the placement of sampling stations in developing a sampling array design. Consideration should also be given to the use and placement of additional particle analyzers for the real-time detection of airborne particles. Strategically placed particle analyzers would be especially helpful to the downwind sampling operator to assist in defining the cross-sectional extent of the plume so that the location of the bioaerosol sampling equipment could be optimized and moved if needed during testing.

6.0 TASK 2. VOLATILE ORGANICS AND ODOR SAMPLING RESULTS

6.1 Objectives

The study evaluated methods that measure concentrations of a selected group of VOCs and odorants in Task 2. Some methods involved sample collection, while others were real-time measurements. Emissions samples were collected upwind, within, and downwind of the application area of the biosolids land application test site. Emissions sampling began prior to biosolids application and continued for 2 days after application. In addition, real-time measurements were conducted on these emissions using VPRM and single path OP-FTIR spectroscopy. OP-FTIR measurements were performed on the day before and the day of biosolids application.

The specific objectives for VOC and odor sampling were to evaluate methods that:

1. Characterize the specific compounds and concentrations of volatile organics and odorants (such as ammonia and hydrogen sulfide) emitted

2. Determine if the VOCs and odorants that were emitted were transported downwind of the biosolids application area.

Several site-specific factors such as wind velocity, atmospheric stability, temperature, humidity, the amount of material being applied, and the particular application method may affect the degree of odor generated at a biosolids application site. Biosolids processing variables also influence biosolids odor (Gabriel *et al*. 2006). This section discusses the various gas monitoring techniques that were evaluated to estimate post-application off-gassing of selected organic and inorganic compounds and the extent to which these selected compounds were observed. Table 2.2b lists the analytes, methods, sample types, and sampling frequencies for the VOC and odor sampling efforts. Analytical methods were evaluated based on whether data acceptance criteria were achieved.

6.2 Volatile Emissions Sampling and Measurements from Biosolids

A selected group of organic, inorganic, and odorous compounds was monitored within various areas associated with this biosolids land application study as described in Section 2.4. Analyses of air emissions were performed on bulk biosolids samples that were collected from the delivery truck as they arrived on site from the treatment plant, from a temporary biosolids stockpile staged near the application site, periodically from the exhaust air of five independent advective flux chambers that each covered a 1.44-m^2 footprint on the surface of the biosolids land application site, and from biosolids samples that were collected from the ground surface approximately 1 hr, 24 hr, and 48 hr after biosolids application. In addition, prior to, during, and following biosolids application, trained odorant professionals surveyed the land application site and surrounding vicinity for the presence of ammonia and sulfide odors using an in-field Nasal Ranger® protocol (ASTM E544-99). The field survey and advective flux chamber sampling locations are shown in Figure 6-1.

6.2.1 Headspace Analysis of Biosolids. The headspace emissions of biosolid samples that were progressively collected from within the on-site stockpile at the time of land application (0 hr) and at 24 hr and 48 hr after application to the field were determined with the use of specially fabricated glass containers shown in Figure 6-2. Each of these three progressive samples was collected in triplicate, with each triplicate sample representing a composite of seven sampling locations. The containers were air tight and equipped with a sealing cap and a septum-sealed sampling port. The samples were refrigerated at 4ºC until analysis. Each sample was allowed to equilibrate to laboratory temperature (20ºC) before

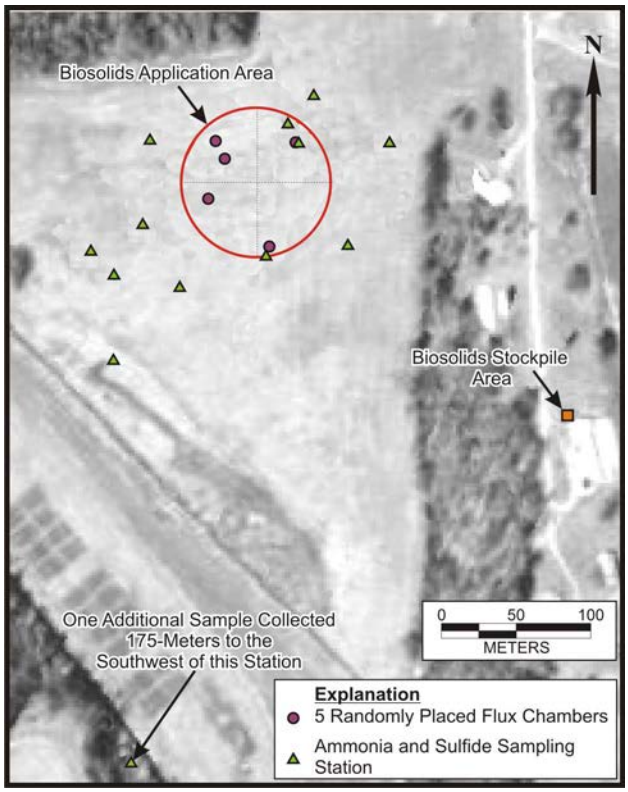


Figure 6-1. Aerial View of Test Site and Sampling Stations

headspace samples were obtained for analysis. A total of 2.0 cm^3 of air was withdrawn from each 100-g (normalized) biosolids sample placed into the sealed container. These headspace samples were analyzed for specific compounds following EPA Method TO-15. An estimated volatile emission factor for each of the three samples was determined using Equation 1:

$$\text{Emission Factor (ng/g)} = \text{concentration (ng/L)} \times \text{volume (L)} \times \text{mass of sample}^{-1}\text{ (g)} \qquad \textbf{(\#1)}$$

6.2.2 Advective Flux Measurements. Five flux chambers (A, B, C, D, and E) were randomly placed within the biosolids application area as shown in Figure 6-1. The flux chambers are discussed in detail in Section 2.4.2, and a conceptual setup is shown in Figure 2-6. Each chamber was constructed of stainless steel, 120 cm on a side and 60 cm high, funneling upward to an opening of 7.6 cm for collection of exhaust that was covered with aluminum foil to prevent downdrafts. Ultra-high purity air was evenly introduced into the bottom of the chamber for make-up air (sweep air) to generate sufficient sample volume. Air samples were pulled into a 5.0-L Summa canister by vacuum and submitted for EPA Method TO-15 analysis by GC/MS. Figure 6-3 shows a flux chamber operating during the post biosolids application sampling event in the field.

Flux chamber emissions were also captured in Tedlar® bags and subsequently analyzed using a SPME absorption technique. The coated fused silica fibers (75-µm carboxen-polydimethylsiloxane fiber) were exposed to the inside of the Tedlar® collection bags for 1 hr for sample equilibrium, then stored on dry ice before being analyzed by GC/MS. The SPME fibers were injected directly into a heated GC/MS port, and the contaminants were thermally released into the GC/MS column. Specific analytes included trimethylamine, carbon disulfide, dimethyl sulfide, dimethyl disulfide, ethyl mercaptan, propyl mercaptan, and butyl mercaptan. Methyl mercaptan was too volatile and reactive to be included as an

Figure 6-2. Glass Vessel Used for Biosolids Headspace Analysis

Figure 6-3. Collecting a Flux Sample in the Field

analyte and also caused calibration concerns. The analyte response for SPME analysis was calibrated using gas standards generated from certified permeation devices (VICI Metronics, Inc., Poulsbo, WA) containing the pure compound.

The estimated flux rate for each of the flux chambers was determined using Equation 2:

$$\text{Flux Rate } (\mu g/m^2/hr) = \text{amount of contaminant collected in SUMMA canister } (\mu g) \times \text{ground surface area of chamber}^{-1} (m^2) \times \text{collection time}^{-1} (hr) \quad \textbf{(\#2)}$$

6.2.3 Off-Site Odor Panel Analysis. Air samples were also collected from the flux chamber emissions and captured in 12.0-L Tedlar® air sample bags via an air pump for odor threshold analysis. A certified odor panel was used to conduct the odor analysis at an off-site forced chamber olfactometer (ASTM, 1991). The sample's dilution level (with air) at which an odor is barely detected from three sources (two are odor free) by a panelist is expressed in the standard units for odor threshold measurement, dilutions-to-threshold (d/T).

6.2.4 Direct Gas Measurements (Ammonia and Hydrogen Sulfide). Field measurements of ammonia were performed using chemical sensory tubes (Draeger Tubes® [Model No. 6733231]) coupled with a hand-operated vacuum pump with a detection limit of 0.100 ppmv. In addition, a direct reading instrument was used for hydrogen sulfide. This gas was measured with a Jerome™ gold-film analyzer (Arizona Instruments) with a detection limit of 0.001 ppmv. Locations were determined in part using Nasal Rangers®, where values determined with the Nasal Rangers® were recorded.

6.2.5 Open-Path Fourier Transform Infrared Spectrometer. An OP-FTIR spectrometer was used to measure "real-time" concentrations of VOC and ammonia emissions from the surface of the land application test site as discussed in Section 2.4.1. The OP-FTIR system was linked to a particle counter that summed the total amount of energy that a target compound absorbed between the FTIR and retro-reflector array. Concentrations of specific compounds were quantified using the measurement of energy absorbed within selected regions of the spectrum.

6.3 Results and Discussion

6.3.1 Head Space Analysis of Biosolids. Detectable levels of acetone, 2-butanone, methylene chloride, toluene, dimethyl sulfide, and dimethyl disulfide were associated with the biosolids that were removed from the stockpile and applied to the field. As shown in Figure 6-4, the estimated emission factor was highest for dimethyl sulfide (range of 227.7 to 658.6 ng/wet g) among the VOCs for all three sampling times (0 hr, 24 hr, and 48 hr). These data represent the arithmetic mean of three discrete field samples, and the error bars indicate the standard deviations of the means. The concentrations for all of the compounds detected decreased for each of the following 2 days, except for dimethyl disulfide, which remained relatively constant or showed a slight increase over time. While methylene chloride was suspected as a laboratory contaminant, the fact that the concentration decreased over time suggests that it also could have been a volatile emission from biosolids. Insufficient data were generated to allow for this differentiation. The other detected compounds are likely organic byproducts of the anaerobic digestion of municipal biosolids typically found in very low concentrations emitting from biosolids as volatile gases.

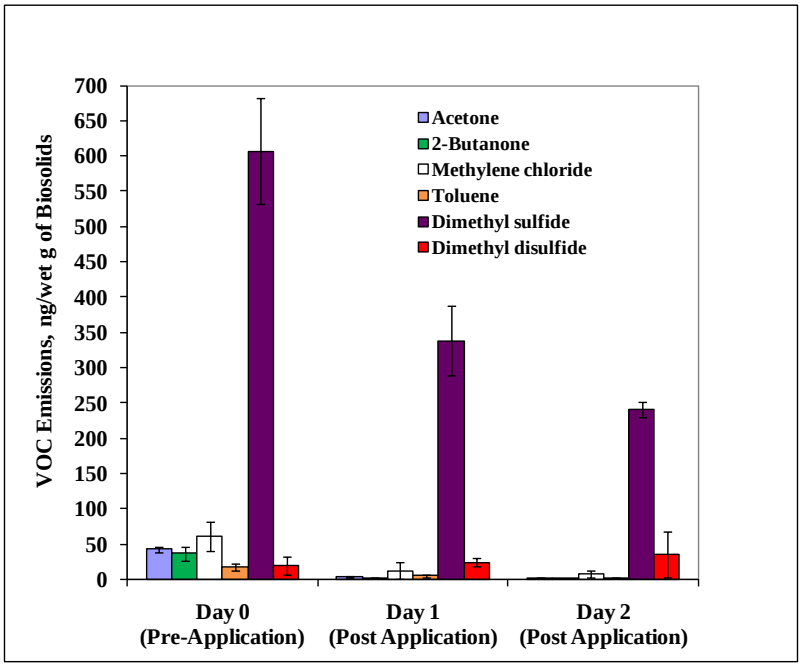


Figure 6-4. Estimated Emission Factors Over Time of Biosolids Application

Results from SPMEs exposed to the headspace of biosolids for 1 hr also resulted in detectable concentrations of dimethyl sulfide (1.75 to 8.0 ppmv) and dimethyl disulfide (0.75 to 2.0 ppmv). Trace levels (0.25 ppmv) of carbon disulfide were also detected in the SPMEs, but only in the control trial test. No significant decreasing trend was observed in the SPME headspace results over the time period (i.e., 1 hr, 24 hr, and 48 hr after biosolids application) that samples were collected.

6.3.2 Advective Flux. The estimated flux rates from the flux chamber air emissions (collected within the Summa canisters and analyzed by GC/MS) resulted in detectable concentrations for acetone, trimethylamine, dimethyl sulfide, and dimethyl disulfide. Estimated flux rates for these compounds were greater than 1.0 $\mu g/m^2/hr$ for several of the post-application sampling times (t = 0 hr, t = 3 hr, t = 4 hr, and t = 20 hr). Figure 6-5 illustrates the flux rates for these compounds at each sampling location. Other contaminants such as isopropyl alcohol, 2-butanone, carbon disulfide, methyl isobutyl ketone, toluene, 2-hexanone, styrene, 1,2,4-trimethylbenzene, and 1,4 dichlorobenzene, were detected at trace levels (2.4 to 3.8 $\mu g/m^3$).

Calculated flux rates for trace compounds are not presented in Figure 6-5. In general, the rate of emissions declined with time after biosolids placement; however, dimethyl sulfide and dimethyl disulfide emissions persisted into the 20th hr after biosolids application when sampling was terminated. A longer monitoring period was needed to determine if emissions continued into the afternoon of Day 2 or if they subsided.

For chambers A through D, the flux rate increased between the 3rd hr and 4th hr after biosolids application. This trend was most likely due to compound volatility caused by increased temperatures during the early afternoon (approximately 2:30 PM). During the 4th hr, internal chamber temperatures reached approximately 42ºC. Since the 4th hr after application was the last time point measured on the day of application, it is not known if the increased temperature in the afternoon continued to enhance emissions.

The SPME apparatus is shown in Figure 6-6. The SPME fibers were exposed to flux chamber emissions captured in Tedlar® bags. Tedlar® bags were collected at each sampling event for all flux chambers and submitted for headspace analysis using the SPME fiber and GC/MS techniques for comparison against the other field methods used for sampling and detecting odorants. However, time did not permit on-site calibration of the GC/MS unit in the field. The results shown here are derived from SPMEs that were exposed to Tedlar® bags in the field and analyzed via a calibrated GC/MS in the laboratory after the field event was completed. Therefore, these data should be considered semi-quantitative as there may have been potential compound losses during extended holding times, and trip blanks were not available to confirm the extent of these potential losses.

SPME results from these field emission tests are shown in Figure 6-7. Dimethyl sulfide was detected in all but two samples, and the levels remained approximately the same, ranging from 0.012 to 0.11 ppmv through t = 20 hr. Dimethyl disulfide was not detected at t = 0 hr and t = 4 hr, but was detected at t = 20 hr (range 0.07 to 0.15 ppmv). Trimethylamine concentrations were highest (range 0.01 to 0.04 ppmv) at t = 0 hr, then decreased. These levels are above the human detection threshold. Carbon disulfide was detected at low levels in all flux chamber samples but was also present in the control trial samples, indicating a potential source independent of the biosolids or possible interference within the Tedlar® bags. Ethyl mercaptan, propyl mercaptan, and butyl mercaptan were not detected.

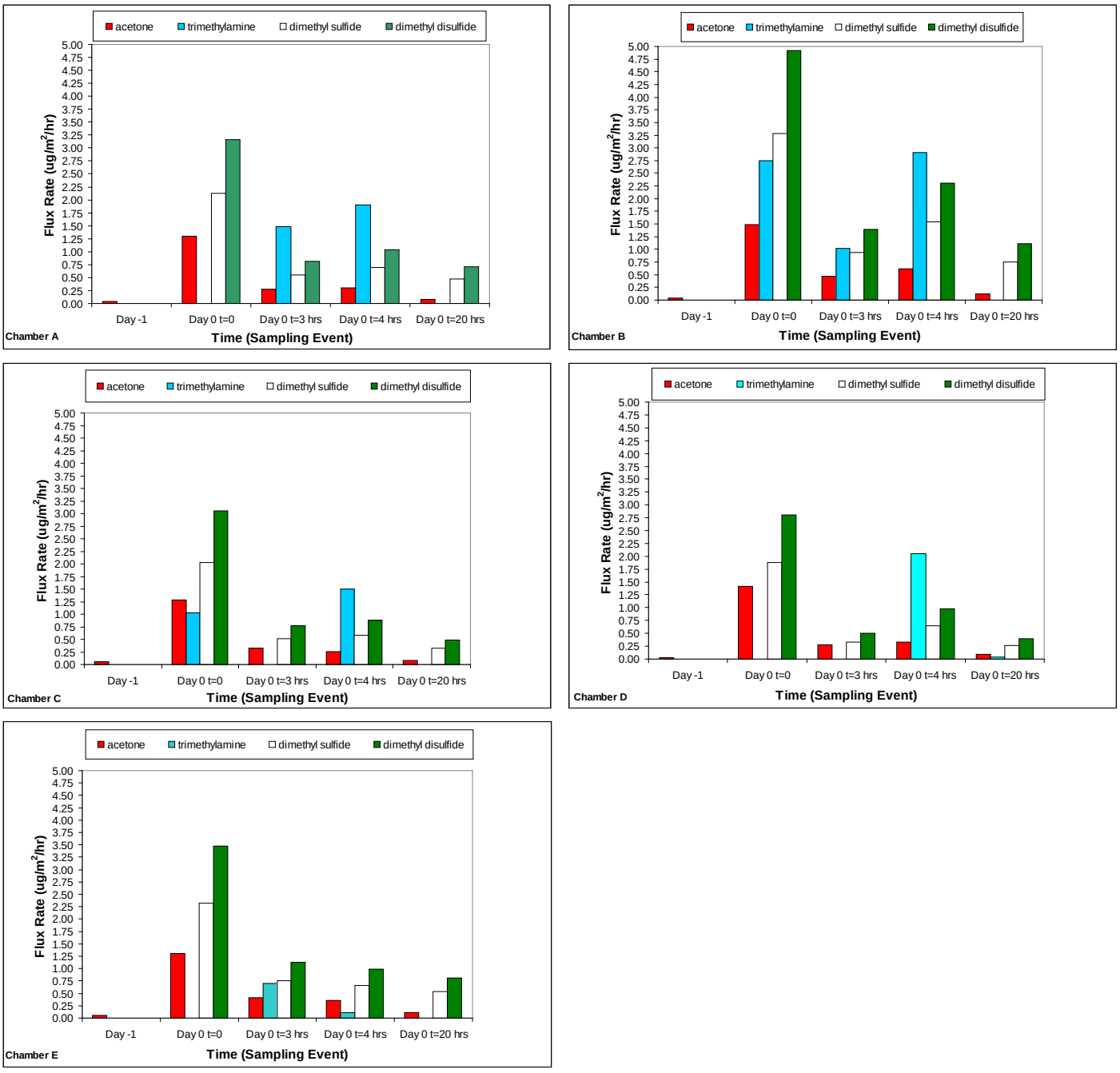


Figure 6-5. Calculated VOC Flux Rates for Acetone, Trimethylamine, Dimethyl Sulfide, and Dimethyl Disulfide for up to 20 hr After Biosolids Application in the Test Area

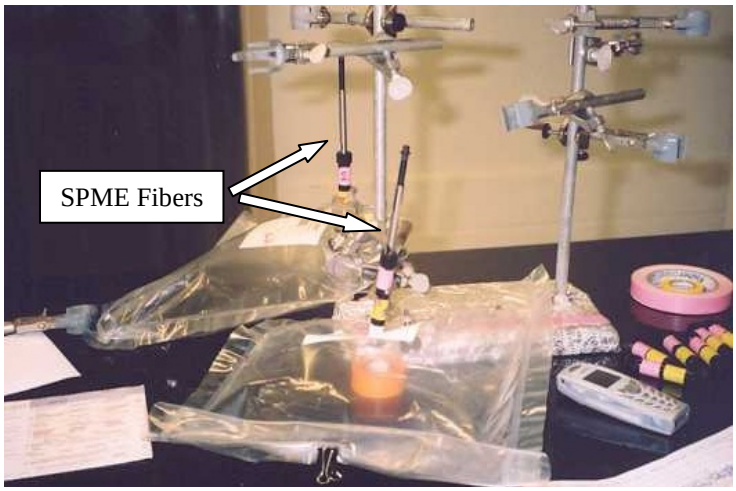


Figure 6-6. SPME Fibers Exposed to Emission Samples Collected in Tedlar® Bags from Flux Chamber Off-Gas

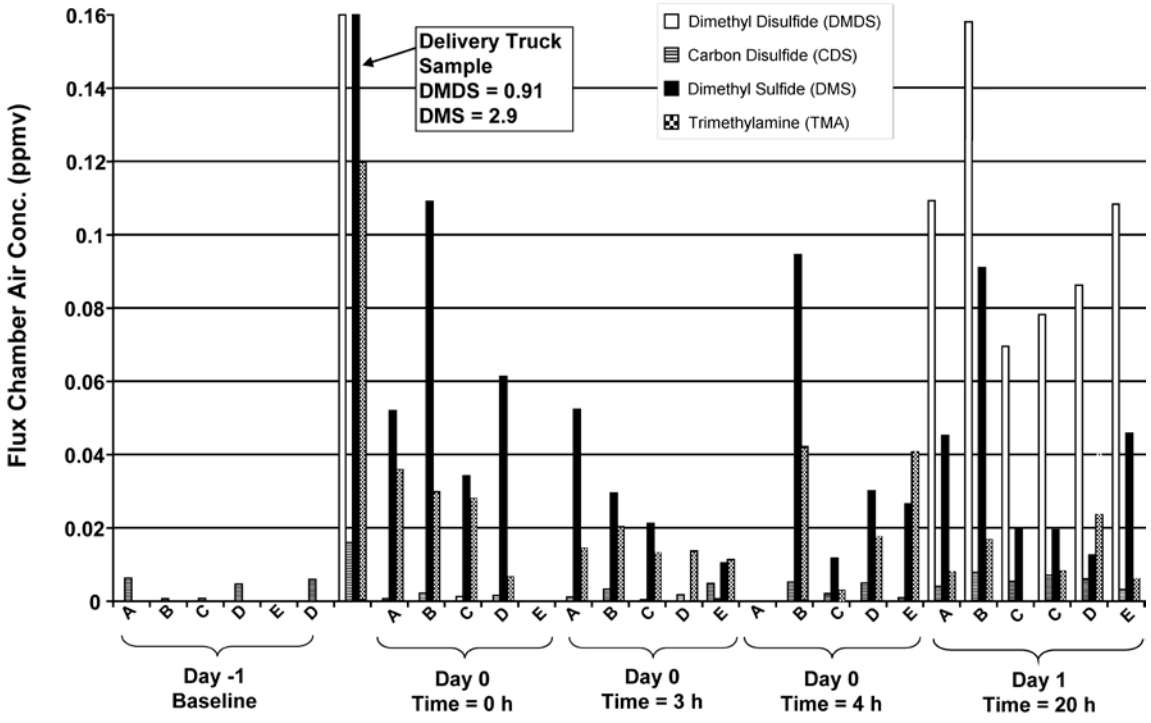


Figure 6-7. Concentration of VOCs from SPMEs Exposed to Air Emissions of Flux Chambers

A comparison of flux chamber emission results between the SPME samples collected in Tedlar® bags and the Summa canister method analyzed by GC/MS revealed that dimethyl sulfide, dimethyl disulfide, and trimethyl amine were found consistently with both methods. Overall, concentration results from the canister method were an order of magnitude higher than with the SPMEs (data not shown), indicating potential losses over time as previously discussed or other interferences resulting in inadequate sorption onto the SPME fiber. On-site processing and analysis of exposed SPME fibers may have produced higher volatile emission concentrations resulting in more comparable results between the two sampling approaches.

6.3.3 Direct Gas Measurements (Ammonia and Hydrogen Sulfide). Ammonia was not detected in above-ground air samples within the application zone area during the control trial. Immediately after the application test, ammonia was detected within the range of 0.10 to 0.90 ppmv for near-ground samples within the application area, and from a flux chamber exhaust sample at 15 ppmv. Hydrogen sulfide was detected at levels near the recognition threshold at concentrations from 0.002 to 0.050 ppmv within the application zone area during the control trial, and at levels of 0.007 to 0.021 ppmv directly behind the moving biosolids application equipment. The exhaust from the biosolids applicator machinery may have been responsible for some of the hydrogen sulfide detected. Immediately after the application trial, hydrogen sulfide was detected at concentrations within the application area slightly lower than those measured during the control trial, within the range of 0.001 to 0.007 ppmv for near-ground air samples, and from a flux chamber exhaust sample at 0.160 ppmv. The highest measurements for each of the gases never approached any health criterion or guidance level. As expected because of their high vapor pressures, the concentration of both gases decreased during the 2nd day (the day after application) to the detection limit, and was below detection limits within 4 days. The concentration of both gases was below detection limits 400 m downwind of the application area during the application trial.

6.3.4 Off-Site Odor Panel and On-Site Nasal Ranger® Analyses. On-site odor measurements were conducted on the biosolids application site using hand-held olfactometers (Nasal Rangers®, St. Croix Sensory, Inc., Lake Elmo, MN). These included measurements (ASTM E544-99 [ASTM, 2004]) made on ambient air at selected locations around the application area. In addition, emission samples for off-site odor analysis (ASTM E679-91 [ASTM, 1991]) were collected from flux chambers B and D in 12.0-L Tedlar® bags via an air pump.

The 12.0-L Tedlar® bag samples obtained by collecting emissions from flux chambers B and D were forwarded to St. Croix Sensory, Inc. for olfactometry analysis by a certified odor panel of three individuals to confirm the presence and level of odor using ASTM E679-91, as described above. In one of three evaluation ports presented to the panel, odor-free air was diluted with increasing levels of contaminated air from the Tedlar® bags. Odor-free air was provided to the other two ports at the same airflow rate. Each panelist was asked to identify the port containing the diluted odor.

The sample dilution level at which odor is first smelled by the panel members is termed the detection threshold (DT). The sample being analyzed by the panel members is further diluted with contaminated air until the panel members recognize the source from a quadrant of odor categories, e.g., rotten cabbage, rotten meat, rotten eggs, strongly fecal, etc. This dilution level is called the recognition threshold (RT).

The unit used in this report to express both DT or RT is dilutions-to-threshold or d/T, where d/T is defined as the volume of uncontaminated odor-free air provided to each panelist at the beginning of the test divided by the volume of contaminated air required to be bled into the uncontaminated air source to reach the respective threshold (either DT or RT). For example, a d/T of 100 is equivalent to one volume of odor-free air divided or diluted by 1/100 volume of contaminated air, while a d/T of 1 is equivalent to one volume of odor-free air divided or diluted by one volume of contaminated air. Note that the smaller

the d/T value becomes the greater becomes the dilution of odor-free air with contaminated air. Odor concentrations for each sample were reported as the geometric mean of the individual panelist's thresholds.

The results for the dynamic dilution and odor threshold analyses performed on Tedlar® bag samples collected from the flux chamber emissions are shown in Table 6-1. DT and RT increased with time indicating that the odors became stronger with time. This is thought to have been due to increasing volatilization resulting from rising temperatures throughout the day, and also because of anaerobic degradation of organic sulfur compounds in the biosolids. For the same sample, DT will always be larger than RT because less contaminated air is needed to detect the odor than to recognize the type of odor.

In Table 6-1, laboratory DT values defined for the control trial (before application) were 70 to 90 d/T. This is a typical background DT range for most rural agricultural areas as measured by a highly-trained, highly-odor-sensitive, off-site panel under controlled laboratory conditions. The panel measured odor DT at 500 to 1,000 d/T from flux chamber sample bags during the first 4 hr. After 22 hr, the panel measured 2,500 to 6,100 d/T from the two flux chamber sample bags. These data suggest that the volatile odors associated with degradation products increased near the ground surface during biosolids application and may increase for a limited time period after application due to ongoing biodegradation and volatilization. Off-site odor panel analyses were not conducted on samples taken more than 22 hr after biosolids application. These odor data are in agreement with observations of increasing concentrations for organic sulfur compounds and other emissions over time shown in Figure 6-7 for samples collected from the same flux chambers.

Table 6-1. Results of Dynamic Dilution Olfactometry Analysis

Flux Chamber	Event	Time (hr)	Laboratory Olfactometry Analysis ASTM E679-91	
			Detection Threshold (d/T)	Recognition Threshold (d/T)
B	Control Trial	NA	70	55
D			90	70
B	After Biosolids Application	0	500	310
D			330	240
B		3	620	330
D			540	290
B		4	620	360
D			1000	540
B		22	2500	1400
D			6100	2500

NA = not applicable

With the exception of the data for flux chamber D at 22 hr, RT values fell within a fairly narrow range of 54% - 79% of the corresponding DT values. This observation indicates the panelists were recognizing odor categories at roughly the same levels of increased dilution with contaminated air after the DTs had been established for nine of the 10 sampling conditions. The ratio of RT-to-DT of 41% for flux chamber D at 22 hr lies outside this range, suggesting either a data outlier or a more difficult-to-recognize odor.

With regard to the odor measurements made on site with the Nasal Ranger® instruments, only ambient trace odor levels were detected at 1.5 m ags within the biosolids application area prior to biosolids application. Immediately after biosolids application, the odor DT levels at 1.5 m ags were 2 to 7 d/T at 25 m upwind of the application area and 15 to 30 d/T approximately 25 m downwind of the application site. Odors were not detected at distances greater than 75 m from the application area. Approximately 22 hr after biosolids application, odor DT levels at 1.5 m ags were roughly 15 d/T in the application area and odor was undetectable above background at levels elsewhere in the project area. After 48 hr, odors were barely detectable downwind of the site, and after 196 hr, no odors were detected above background in any location on the site, consistent with other biosolids application studies (Krach *et al*., 2008; Hamel *et al*., 2004). As expected due to the high rates of air dispersion associated with measurements made in ambient air, the odor measurements decreased vertically above ground surface (ags) and horizontally at increased distances from the application area. The downwind values may have been greater if the biosolids application area was larger or if the biosolids loading was greater.

As opposed to the pristine laboratory conditions under which off-site odor measurements are conducted, on-site odor measurements are carried out by roving observers or receptors using hand-held olfactometers substantially less sensitive than the trained human nose. In addition, on-site observers are subjected to background odors to which the off-site panel are not exposed. Understandably, then, the on-site DT values determined with the Nasal Rangers® were substantially less than those measured for the flux chambers by the off-site panel. Also, as expected, the measured off-site DT values decreased with time after biosolids application as odors were dispersed, rather than increased with time as did the levels measured in the flux chamber emissions due to enhanced volatility and biodegradation of trapped organic compounds. While the Nasal Rangers® are not as sensitive as the off-site panel, the results obtained with these instruments should be considered the more valid data set for accurately representing on-site odor conditions to an on-site human receptor.

6.3.5 Open-Path Fourier Transform Infrared Spectrometer Measurements. VRPM was performed by deploying 10 mirrors in various locations on a vertical plane in line with the scanning OP-FTIR sensor. The vertical plane was configured as close to perpendicular to the prevailing wind direction as possible. By combining measured wind data with the path-integrated concentration data, the emission flux through the vertical plane was calculated. Two VRPM configurations were used, one upwind and one downwind. Each VRPM configuration consisted of five mirrors, three placed along the surface and two mounted on a vertical structure (scissorjack) positioned approximately 8 m and 12 m ags. A Midac™ OP-FTIR was used in the upwind configuration, and an IMACC™ OP-FTIR was used in the downwind configuration. See Figure 2-5 for the on-site configuration that was used to deploy the OP-FTIRs.

An additional IMACC™ OP-FTIR was deployed along a single path (one-way path length of approximately 141 m) over the center of the application area. Data from this instrument were collected to measure ammonia concentrations and determine whether VOCs were present in the application area. No interpretive data were found relative to VOCs and, therefore, are not presented in this report.

The ammonia measurements were collected on Day -1, Day 0 during application, and Day 0 for several hours after application. On Day -1, baseline measurements helped to determine the VOC contribution from various generators and equipment and from the tractor during the baseline sampling event. On Day 0, measurements were collected during the entire application. Measurements continued for several hours after the application ended to evaluate the decrease of emissions over time.

6.3.5.1 Baseline Ammonia Measurements. During Day -1, baseline measurements were collected along each of the VRPM configurations. The upwind VRPM configuration failed to detect the presence of any ammonia plumes during the entire duration of this measurement period. The downwind VRPM

configuration measured negligible ammonia concentrations throughout the duration of the baseline measurements.

6.3.5.2 Ammonia Measurements During Biosolids Application. During the period of biosolids application, measurements were collected along each of the VRPM configurations. The VRPM procedure calculates the concentration values for every square elementary unit in a vertical plane. Then, the VRPM procedure integrates the values, incorporating wind speed data at each height level to compute the flux. The concentration values are converted from parts per million by volume (ppmv) to grams per cubic meter (g/m^3) taking into consideration the molecular weight of the target gas. This enables the direct calculation of the flux in grams per second (g/s) using wind speed data in meters per second (m/s).

The calculated ammonia flux from the upwind VRPM configuration during and immediately after biosolids application was 0.006 g/s. Figure 6-8 shows the reconstructed ammonia plume for the upwind VRPM configuration. The calculated ammonia flux measured from the downwind VRPM configuration for the same time period was 0.063 g/s. The reconstructed ammonia plume from these measurements is shown in Figure 6-9. The bold vertical lines in Figures 6-8 and 6-9 and in the figures to follow in Section 6.3.5.3 represent the physical height of the retro-reflectors on the ground and in the tower. The angled lines projecting from the origin indicate the path length. The positioning of the retro-reflectors and the path lengths is illustrated for clarity in Figure 6-8.

Upwind ammonia concentration contours are shown in Figure 6-8. Concentrations were greatest near the ground and in the immediate vicinity of the biosolids application area at approximately 0.018 ppm and dissipate radially to 0.004 ppm at a height of approximately 14 m and over a horizontal span of approximately 95 m.

The ammonia concentration plume in the downwind configuration (Figure 6-9) is dispersed over greater horizontal and vertical distances (approximately 21 m ags and 180 m laterally). The ammonia concentrations were an order of magnitude greater than those for the upwind configuration at 0.15 ppm near the ground and 0.03 ppm for the most distant radial contour.

6.3.5.3 Ammonia Measurements After Biosolids Application. In order to investigate the rate of emissions decay, measurements continued for several hours after biosolids application. The upwind VRPM configuration measured negligible ammonia concentrations during the post-application period, and, therefore, the results are not shown graphically. However, the downwind VRPM configuration detected ammonia plumes for several hours after the application ended. Figures 6-10 and 6-11 depict reconstructed ammonia plumes in the downwind location at 2 and 3 hr, respectively, after biosolids application.

The calculated emission flux rates for ammonia 2 and 3 hr after biosolids application were approximately 0.036 and 0.009 g/s, respectively. There is an observed decrease in ammonia emissions over the 2 to 3 hr time interval.

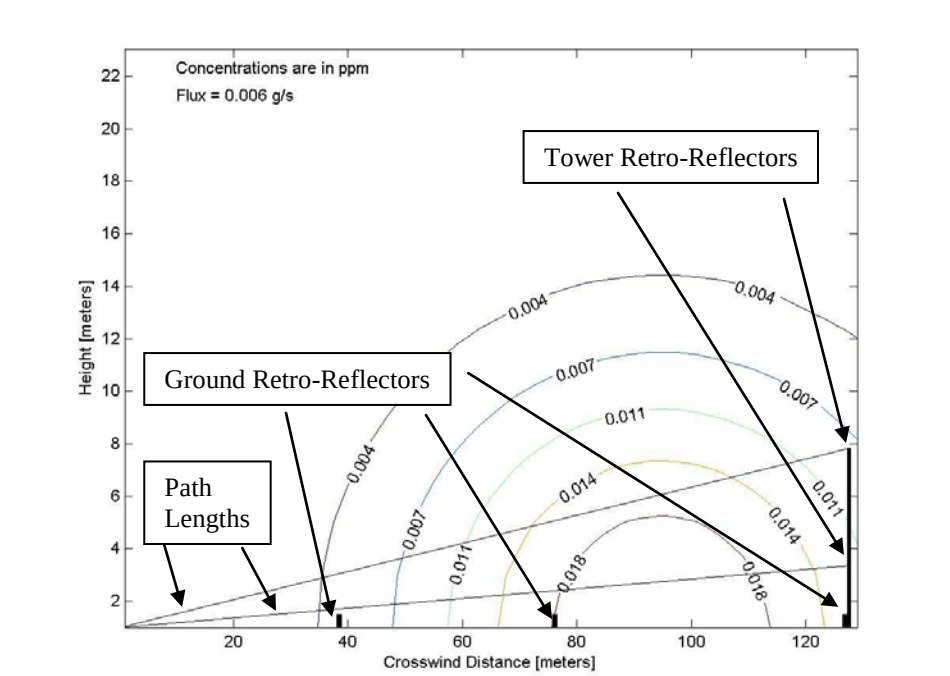


Figure 6-8. Reconstructed Ammonia Plume from the Upwind VRPM Survey During Biosolids Application on Day 0

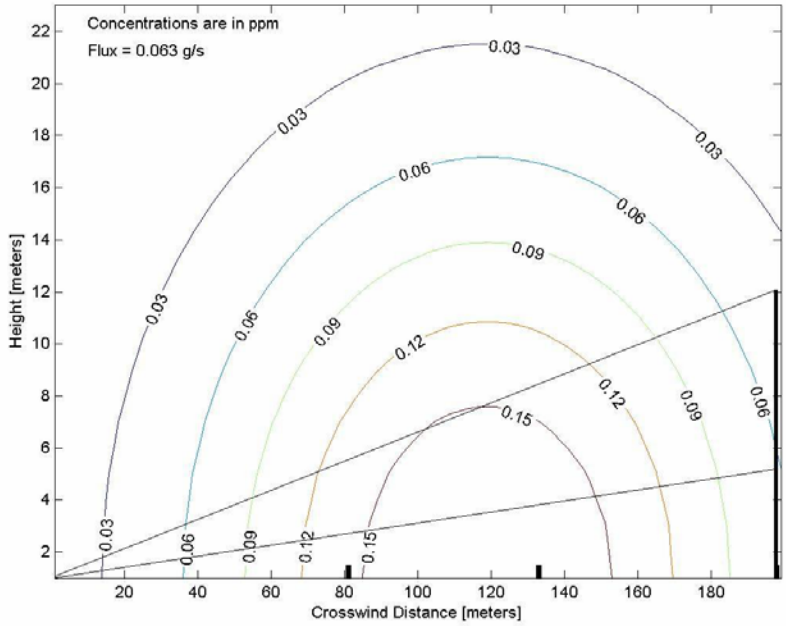


Figure 6-9. Reconstructed Ammonia Plume from the Downwind VRPM Survey During Biosolids Application on Day 0

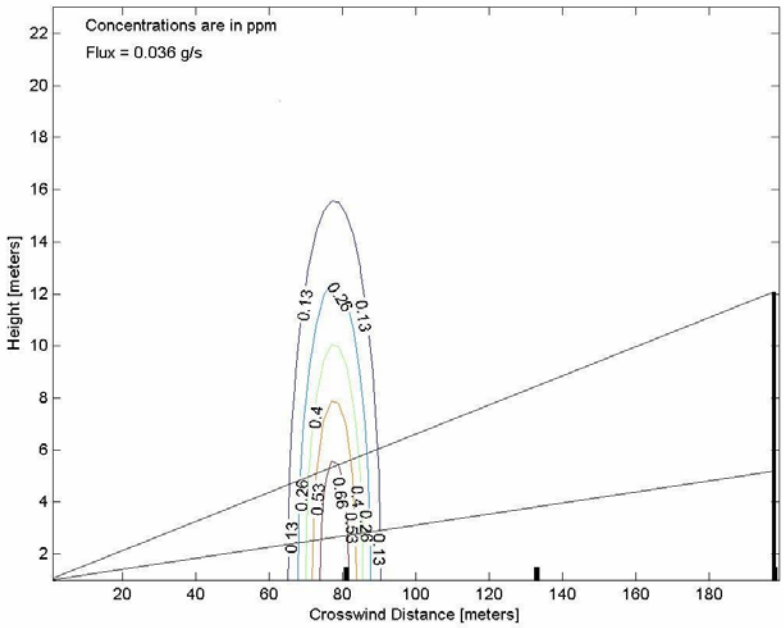


Figure 6-10. Reconstructed Ammonia Plume from the Downwind VRPM Survey Approximately 2 hr After Biosolids Application

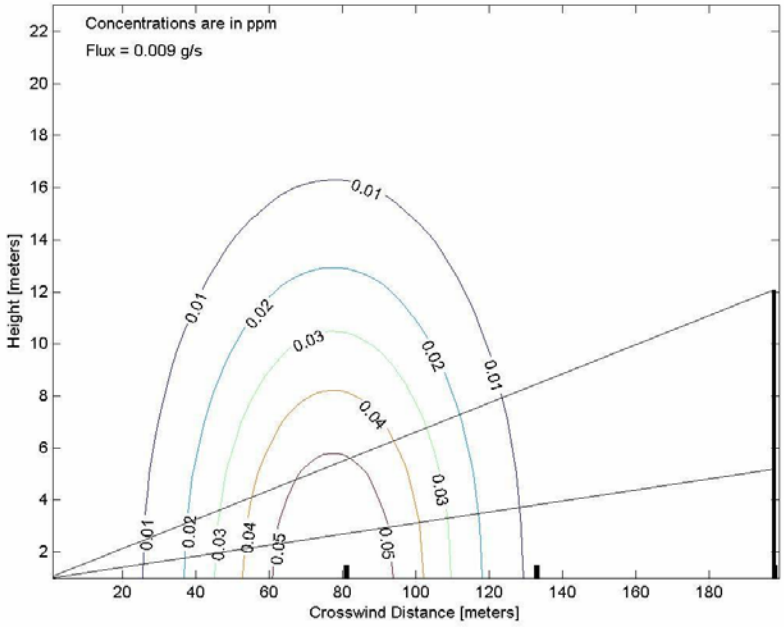


Figure 6-11. Reconstructed Ammonia Plume from the Downwind VRPM Survey Approximately 3 hr After Biosolids Application

The height of the downwind ammonia plume decreased to approximately 16 m ags 2 hr after application from an initial height (t = 0 hr) of approximately 21 m and maintained this height at 3 hr. The lateral plume dimension at 2 hr after application decreased to approximately 25 m in width. At 3 hr after application, the lateral extent of the plume unexplainably expanded to a width of 100 m but decreased to a concentration that was an order of magnitude less than that observed at 2 hr.

6.4 Conclusions

During and after biosolids application, various organic and inorganic odors were detected above background levels approximately 1.5 m ags and in concentrated samples collected from flux chambers. Analysis of air samples collected from flux chambers using GC/MS confirmed that odors were primarily associated with compounds such as dimethyl sulfide, dimethyl disulfide, and trimethylamine. Acetone, which was quantified by GC/MS, is not believed to be a common constituent of biosolids or their biodegradation breakdown byproducts; however, it appeared at relatively high flux rates (1.25 $\mu g/m^2/hr$) in flux chamber samples. SPME analysis of headspace samples that were equilibrated with biosolids confirmed the presence of dimethyl sulfide, dimethyl disulfide, and trimethylamine at approximately an order of magnitude less than those concentrations determined through GC/MS analysis.

Volatilization and further degradation of biosolids resulted in increasing detectable concentrations of odors captured in flux chamber emission samples for 3 to 22 hr after application. Biosolids application also increased the near-surface concentration of hydrogen sulfide and ammonia immediately after application, but concentrations at 1.5 m ags were similar to background conditions, as observed in Draeger Tube® sampling. Concentrations of ammonia and hydrogen sulfide at ground level began to decrease within 3 to 4 hr after application, then to non-detectable levels within 20 hr.

OP-FTIR results confirmed the in-field detection of ammonia collected via Draeger Tube® and Nasal Ranger® measurements. The VPRM protocol showed a decreasing ammonia emission flux rate, initially measured at 0.063 g/s, decreasing to 0.036 g/s approximately 2 hr after the application ended, and further reducing to 0.009 g/s 3 hr after the application ended.

6.5 Discussion and Lessons Learned

While odor was virtually undetectable using Nasal Rangers® 2 days after biosolids application at this site, and observed still to be undetectable above background levels at 4 days after application, it is not known to what extent changing weather patterns may have impacted this apparent trend and whether or not odors may have "rebounded" after monitoring was terminated. Furthermore, differences were observed in environmental conditions between the control trial and the biosolids application test that may have influenced volatilization results. For the control trial (Day -1), which occurred between the hours of 2:07 PM to 3:35 PM on September 29, 2004, ambient air temperature was approximately 25ºC, relative humidity was 50%, and the solar index was 644 w/m^2. These values are compared to the application test (Day 0) where biosolids application was conducted between 9:37 AM and 11:26 AM, the ambient air temperature was approximately 20ºC, the relative humidity was 86% (with a visible light fog), and the solar index was 404 w/m^2.

Although the use of chambers seemed to be an effective approach for measuring flux emissions, the elevated temperatures inside the chambers most likely increased the volatility of the organic compounds measured and may have enhanced microbial activity on the ground. Also, in retrospect, a more focused sampling schedule with better resolution between 4 and 22 hr after biosolids application, combined with other weather effects data, would have provided a better understanding of the extent of volatile emissions and the recalcitrance of particular compounds.

Lastly, the post-application field sampling efforts in the application zone associated with this task and other tasks for this study created logistical challenges. Activity in the field from vehicles used to transport samples and equipment in some instances obstructed the OP-FTIR pathway to reflectors positioned on the ground surface and may have created aerosols that were introduced into the beam path. A modified design should be considered for future studies that reduces or eliminates these types of interferences. Development of such a design is beyond the scope of this project, but most certainly would begin with a reduction in the number of simultaneous sampling activities that can lead to interference.

7.0 TASK 3. LAND SAMPLING RESULTS

7.1 Objectives

The soil sampling portion of this study focused on methods to measure the concentrations of microbes and chemicals before and for 98 days after biosolids application. The specific objectives for Task 3 were to evaluate methods that:

1. Characterize the quantity and distribution of biosolids applied
2. Characterize the microbial community quantity and structure
3. Measure the fecal coliform density
4. Measure the concentration of alkylphenol ethoxylates (APEs) and their degradation products
5. Screen samples for terrestrial ecotoxicity.

Several concepts were considered in selecting these objectives. Objectives 1 and 2 were identified to better describe observations of this study. Biosolids application methods can vary considerably. One objective was to evaluate a method to measure the quantity and distribution of biosolids applied at this site. Biosolids distribution was determined by measuring the dry mass and ash mass of applied material for each replicate plot. These data were evaluated for consistency between plots and for spatial variation within a plot. PLFA measurements were used to characterize the size and structure of the microbial community. Biosolids may alter the microbial community by adding nutrients, organic matter, and microbes. PLFA data provided insight into the magnitude and diversity of these changes. Objectives 3 and 4 were based on specific recommendations of the 2002 NRC report to study the impacts of pathogens and chemicals, such as the surfactants used in cleaning products and detergents. Fecal coliforms were measured as potential indicators of pathogenic bacteria. The NRC report (2002) expressed concern about the persistence of organic compounds in environmental matrices and the potential for transport within soils and to other environmental media. Surfactants, such as APEs, are produced and used in large volumes. These compounds and their degradation products, such as octylphenol (OP) and nonylphenol (NP), have been reported to disrupt endocrine activity. Objective 5 was included to evaluate whether biosolids introduce toxicity based on observed responses in some ecologically relevant organisms. Soil toxicity was screened using a 14-day earthworm mortality bioassay and a 5-day assay measuring seed germination and root elongation in lettuce and oats. One stated reason to land apply biosolids is to serve as a soil amendment, increasing the soil organic matter and supplying nitrogen and phosphorus to enhance plant growth. In this study, observed responses were compared before and after application to evaluate whether biosolids application improved growth or survival in these toxicity assays.

Data collection was not limited to information needed to meet study objectives. For example, supporting information such as soil agronomic characterization, temperature, and weather data were gathered. In addition, microbial indicators such as VHO, *Salmonella*, *Enterococcus* spp., THB, enteric viruses, and male-specific coliphage were analyzed on selected samples.

This section discusses the data collected and, where possible, interpretation of results and recommendations for future studies. Table 2-2d lists the analytes, methods, sample types, and sampling frequencies for the land sampling effort. Analytical methods were evaluated based on whether data acceptance criteria specified in the QAPP were satisfied. When data quality was adequate, further analysis was conducted to interpret the data. Recommendations were made to improve methods and

sampling techniques in the discussion of the data sets. Recommendations for future studies were also included.

7.2 Overview of Field Plots

For this field-scale research project, a research material comprised of anaerobically digested biosolids mixed with lime and dewatering additives was applied at agronomic levels to a fescue field (see Section 3.0 for more information on biosolids application). Biosolids had not been applied to this field in the past. This material was applied in a 100-m diameter circle by a side discharge manure spreader. The soil sampling occurred in three replicate plots of 3 m across by 6 m long, randomly located within the biosolids application area (see Figure 2-7). All samples were collected based on a predetermined randomized sampling plan. In the first half of each plot (3 m by 3 m), the distribution of biosolids at Day 0 (day of application) was measured. Ecotoxicity samples were also collected in this section. In the second half of each plot (3 m by 3 m), the soil was sampled at three selected locations (30 cm by 30 cm) for each sample event using a grid system. In each location, multiple samples were removed, but each location was sampled only once. Soil samples were collected from the three replicate plots prior to biosolids application (two sample events) and for 98 days following application (five sample events). The post-application sample period was selected based on site restrictions for fields where Class B biosolids have been applied. Site restrictions are dependent on whether biosolids are allowed to remain on the surface or are incorporated within a 4-month period. Thus, the sample period was selected to be slightly less than 4 months. Sample collection methods, sample events, sample depths, and sample compositing varied depending on the analyte.

7.3 Sample Collection and Analysis

7.3.1 Collection Methods. For most land sampling activities, a coring method was used to obtain the sample. Biosolids distribution and ecotoxicity samples were collected using different methods. Coring equipment consisted of a 60 cm stainless steel split spoon sampler (6.3-cm diameter) that was driven with a hydraulic hammer to the appropriate depth based on the analyte of interest for that sample. Specific sample locations within a grid were selected to include biosolids based on visual inspection after biosolids application. The spoon was pulled out of the soil using a core pulling device, and the core sample was retrieved by opening up the split spoon and processing the soil core to remove the specific depth intervals using a decontaminated and sterile putty knife. Soil segments were transferred into clean glass or plastic jars. Sterilized sample jars were used for samples that were to undergo microbiological analyses. Split spoon samplers were sterilized by autoclave prior to use in the field. Samples were shipped for analysis.

Biosolids distribution was collected by securing 30-cm × 30-cm squares of geotextile to the soil surface at preselected random locations prior to biosolids application. Within 1 hr after application, the squares were lifted off the soil and any biosolids on the surface of the square (along with the geotextile) was placed in a sample bag. Samples were shipped for analysis of dry mass, ash mass, and volatile solids.

A sample collected for measuring ecotoxicity was generated by compositing soil samples from four preselected random subsample locations in a plot. Each subsample was a soil cube of 15 cm × 15 cm × 15 cm (L×W×D). For each sample, the subsamples were placed in a single bucket and shipped for analysis.

Weather data were collected on site using an HOBO Weather Station Model H21-001. Prior to biosolids application, the weather station was placed adjacent to the field site. Immediately after biosolids application, the weather station was placed on the application area next to Plot 3. The weather station monitored parameters that could affect the study variables, including rainfall, soil water content/moisture, soil temperature, air temperature, relative humidity, dew point, and solar radiation.

7.3.2 Analyte Specific Sample Collection and Analysis Information. Table 7-1 lists the sample events and the number of samples collected for each analyte. The pre-application soil sampling event took place 3 days before air sampling in order to avoid confusion in logistics with the air sampling team.

Biosolids distribution was measured on the day of application as described in Section 7.3.1.

Sample collection for PLFA analysis used the coring technique. In each plot, PLFA samples were collected at three locations and at three depths (0 to 5, 10 to 15, and 20 to 25 cm) for each location. Samples were not composited. Thus, each sample event generated a total of 27 PLFA samples.

Table 7-1. Sample Analytes, Events, and Sample Numbers for Land Sampling

	Sampling Event							
	Soil							Biosolids[a]
Analyte	Day -35	Day -3	Day 0	Day 14	Day 28	Day 63	Day 98	Day 0
Biosolids dry mass/volatile solids	NS	NS	60	NS	NS	NS	NS	NS
FAME	27	27	27	27	27	27	27	3
Fecal coliforms[(b)]	9	9	9	9	9	9	9	3
APEs, Bisphenol A	27	27	27	27	27	27	27	3
Soil characterization	9	NS	9	NS	9	NS	9	1
Ecotox	3	NS	3	NS	NS	NS	3	NS
Microbial indicators[(c)]	3	3	3	3	3	3	3	3
Microbes[(d)]	3	NS	NS	NS	3	NS	3	3

NS = no sample collected

(a) A composite sample formed by combining seven subsamples from random locations in the biosolids pile prior to biosolids being applied to land.

(b) MPN method analyzed by Environmental Associates, Inc.

(c) Sent to Environmental Associates, Inc. for VHO, *Salmonella*, enteric viruses, and male-specific coliphage analysis.

(d) Sent to USDA for total heterotrophs, fecal coliforms, *S. aureus*, *Enterococcus* spp., *E.coli*, and *C. perfringens* analysis.

Samples collected for fecal coliform analysis used the coring technique and sterile equipment. In each plot, these samples were collected at three locations at the 0 to 5 cm depth. Thus, each sample event generated a total of nine fecal coliform samples.

Samples for measuring APE concentrations were collected using the coring technique. In each plot, these samples were collected at three locations and at three depths (0 to 5, 10 to 15, and 20 to 25 cm) per location. APE samples were not composited. Thus, each sample event generated a total of 27 APE samples.

Similarly, the coring technique was used to collect soil characterization samples. In each plot, these samples were collected at three locations and at three depths (0 to 5, 10 to 15, and 20 to 25 cm) for each location. These samples were composited based on depth. For example, the three 0 to 5 cm samples from a plot were mixed together. Thus, each sample event generated a total of nine soil characterization samples.

Samples for ecotoxicity measurements were collected as described in Section 7.3.1 and in accordance with the sampling schedule (Table 7-1). At each sample event, a total of three composite samples were collected.

Samples for microbial indicator and microbe analysis were collected using the coring technique and sterile equipment. Three cores were collected from a plot, and the 0 to 5 cm portions were composited to form a microbial indicator sample. This procedure was repeated in each plot. Thus, a total of three microbial indicator samples was generated at each sample event listed in Table 7-1. Samples collected to quantify specific groups of microbes (microbe samples) were collected in a similar fashion.

Additional information about sample collection, compositing, and analysis was specified in the QAPP and is discussed in later sections.

7.3.3 Statistical Analysis. The same general statistical approach was used in the analyses of the data for Objectives 1, 3, 4, and 5. General linear analysis of variance (ANOVA) models were fitted to the data for each study component, and a backward selection method was used to reduce the terms in the model. In backward selection, insignificant variables are removed from the model one at a time, starting with the least significant, until only significant variables or variables of interest remain in the model. For the objectives where more than one variable was measured (dry and ash mass, APEs, and soil characterization data), multivariate ANOVA (MANOVA) models were used to account for correlation between the measured variables. In addition, the distribution of the model residuals was explored after each model was fitted to determine the extent to which the underlying assumptions of ANOVA were met. In general, interactions between the factors were included in the model, where appropriate. The predictive factors that were included in the models for each analysis were:

- Biosolids distribution - plot, row, and column locations (separate models fitted to each plot)
- Biomass by PLFA – time, plot, depth, and two-way interactions
- APE concentration - time, plot, depth, and time-by-plot interactions
- Fecal coliform density - time, plot, and time-by-plot interactions
- Terrestrial ecotoxicity - time, plot, sample soil concentration, and all two-way interactions.

For the root elongation component of the ecotoxicity data, separate models were fitted for lettuce and oats.

For those analyses where predictive factors were found to be statistically significant, multiple comparison procedures were applied to determine the specific ways in which the measured variable was affected by the significant factor. When time was a significant factor, the multiple comparison analyses compared post-application levels to pre-application levels (controls) as well as comparing among post-application levels. For other factors, all factor levels were compared. Multiple comparisons were performed using Scheffè's method to control for overall error rate among all possible contrasts. For those analyses where there were statistically significant interactions, multiple comparisons were performed by fixing one of the interacting factors at each of its levels (e.g., fixing depth at each of its three values) and comparing the means among the levels of the second interacting factor.

For the microbial community structure part of Objective 2, the PLFA data were subjected to multivariate data analysis in order to characterize the relationships between the samples by category prediction methods. Hierarchal cluster analysis (HCA) and principal component analysis (PCA) were the two exploratory methods used in examining this dataset. All PLFA data were transformed to a mass percent basis prior to analysis. HCA organized samples by similarity (least distance). The methods of HCA used in this study were incremental, centroid, group average, and median. The incremental method

of HCA gave the deepest branching and tightest clusters. PCA was run with preprocessing by mean-center, maximum factors of 10, no rotation, and cross validation. The scores and loadings plots were examined for the presence or absence of groupings and for the possibility of outliers. If outliers were suspected, then an outlier diagnostic was performed by plotting sample residuals vs. Mahalanobis distance (Beebe, *et.al*., 1998). If the sample was more than two threshold values away, it was removed as an outlier, a new PCA was performed, and the process was repeated.

7.4 Data and Results

7.4.1 Soil Characterization. Soil characterization data collected throughout the study, available in Appendix D, were used to define the conditions at this site. Statistical analysis identified differences in soil properties with depth, plot, and time as well as interaction effects between plot and depth. Differences by depth were observed in most soil characterization measurements, followed by differences between plots. Statistical differences based on sample time were observed in six measurements, but these differences did not exhibit consistent trends (such as an increase or decrease after biosolids application). Similarly, interactions between plot and depth were observed in a few soil characterization measurements, but did not exhibit consistent trends.

Soil properties data are summarized in Table 7-2. Since the depth parameter showed the most differences, data have been grouped based on depth. For example, statistical analysis identified that the bulk density was different in each horizon; therefore, averages and standard deviations have been calculated for each depth. For cation exchange capacity, the surficial depth was distinct from the deeper samples, so data were averaged in two groupings (0 to 5 cm depth range, or the combined 10 to 15 and 20 to 25 cm depth ranges). For each measurement type, the final column lists other variables for which statistical differences were observed.

Table 7-2. Soil Characterization Data for Land Sampling Plots

Soil Measurement	Biosolids	Soil Depth			Other Statistical Differences[c]
		0-5 cm	10-15 cm	20-25 cm	
Soil Composition					
Sand (%)	5.0	40.7 ± 6.5		30.7 ± 7.0	T
Silt (%)	78.6	24.8 ± 5.7			P, DP
Clay (%)	16.4	34.5 ± 6.5		44.5 ± 8.4	P , T
Bulk density (g/cm^3)	0.89	0.84 ± 0.05	1.04 ± 0.03	0.99 ± 0.06	T, P, DP
Cation exchange capacity (meq/100 g)	22.0	12.4 ± 0.6	10.2 ± 0.8		P, T
Moisture at 1/3 bar (%)	158.4	33.7 ± 3.2[b]	26.1 ± 1.7	33.25 ± 4.6[b]	DP
pH	7.4	6.0 ± 0.2	6.7 ± 0.7	6.8 ± 0.2	P, DP
Organic matter (%)	NA[a]	7.2 ± 1.1	1.9 ± 0.2	1.2 ± 0.2	
Total N (%)	NA	0.412 ± 0.500			T, DP
Total P (mg/kg)	24453	1152 ± 114	447 ± 44	322 ± 28	P
Olsen P (mg/kg)	176	43 ± 6	6 ± 2		T
Soluble salts (mmhos/cm)	4.09	0.24 ± 0.13	0.11 ± 0.04		DP

(a) NA not analyzed
(b) The two depth ranges 0 to 5 and 20 to 25 cm are statistically similar.
(c) Other statistical differences for soil measurements: P, plot; T, time; and DP, interaction of depth by plot.

Soil characterization data were useful in documenting conditions at this site, and similar data collection efforts are recommended for other sites. If budgets do not permit data collection at this level, soil characterization efforts could be reduced. In this study, shallow samples displayed more dynamic effects.

7.4.2 Weather Data. Rainfall and soil moisture data are presented in Figure 7-1. A substantial rainfall occurred immediately prior to biosolids application. In addition, several smaller rain events occurred throughout the study. Consequently, the soil moisture remained close to water holding capacity throughout the study and, thus, desiccation was not an issue in this study.

Figure 7-2 displays the soil temperature data from Day -3 (September 27, 2004) to the end of the sampling period. In this graph, sample events are noted by arrows. The horizontal line marks 8°C, the upper temperature limit for holding microbial cultures. Temperatures declined throughout the study and were low during the last sample events. The lower temperature may have affected the observations for this study. For example, degradation of APEs may have been retarded by colder temperatures.

Weather data collection was also useful in documenting site conditions. Based on experience at this site, similar data collection efforts are recommended for other studies. If budgets permit, it would be useful to collect soil moisture and temperature data for each replicate plot since plot-to-plot variations were seen for some measurements in this study.

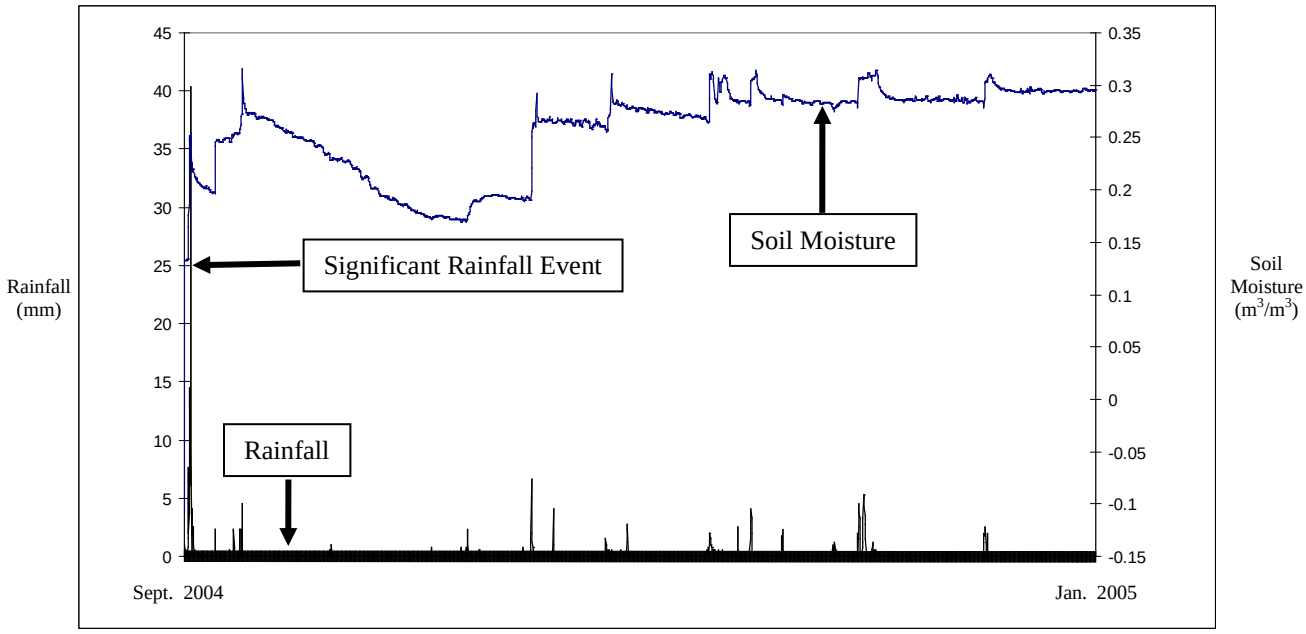


Figure 7-1. Rainfall and Soil Moisture Measurements During Land Sampling Period

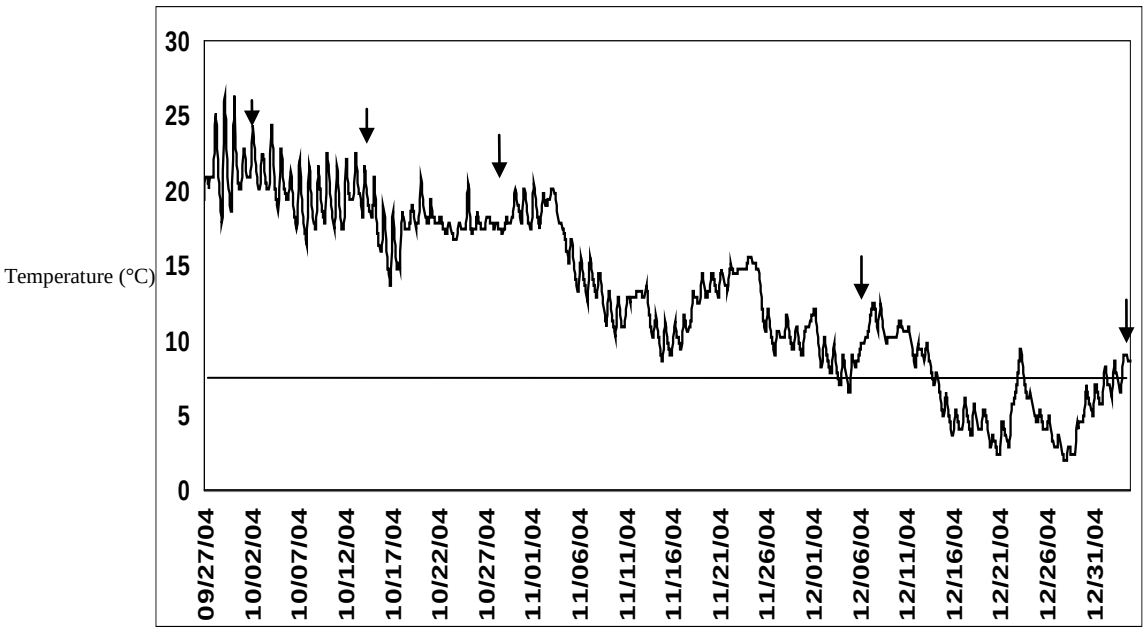


Figure 7-2. Soil Temperature During Land Sampling Period. (The probe was placed 5 cm beneath the soil surface. Arrows indicate sampling events. The horizontal line marks 8°C, the upper temperature limit for storing microbial samples.)

7.4.3 Biosolids Distribution. The goal of measuring the quantity and distribution of biosolids after application was to characterize biosolids application for this study. Two photographs are presented in Figure 7-3. In A, the squares of geotextile were pinned to the soil prior to biosolids application. In B, a post-application photograph is shown. Twenty squares were randomly positioned in each plot. Each sample was analyzed for the wet, dry, and ash mass of biosolids on each 900 cm^2 square of geotextile. Analysis met all data quality objectives from the QAPP.

One-factor ANOVA models were fitted to three measurements (dry mass, wet mass, and ash mass) to determine if significant differences existed among the three plots. These analyses showed that Plot 2 received significantly higher levels than Plots 1 and 3 for all three measurements. A boxplot of the ash mass data illustrates this observation in Figure 7-4.

To determine whether spatial variability was observed within plots, a separate ANOVA model was fitted to the three measurements for each plot. The model included predictive factors for both the row and column locations (see Figure 2-7). It was not possible to include interaction between these factors because there were too few observations (less than 20) to estimate differences between 20 different factor levels (10 rows and 10 columns). For Plots 1 and 3, the three measured variables were not affected by either the row or column locations. For Plot 2, both the row and column variables had significant effects on all three responses. This analysis showed that biosolids application was even in Plots 1 and 3 and uneven in Plot 2.

The statistical observations regarding spatial variability in Plot 2 were consistent with field observations. During application, the spreader applied biosolids aerially, but a trail of biosolids was left from one side of the spreader. This trail left a banding pattern across the application area as seen in Figure 7-5. The spreader drove diagonally across Plot 2, and the trail of biosolids, visible in Figure 7-5, may have contributed to the uneven distribution observed.

Figure 7-3. Photographs of Land Sampling Plots Before and After Biosolids Application: A – Before Application (black squares are geotextile used to measure biosolids distribution); and B – After Application (These photographs were from different plots.)

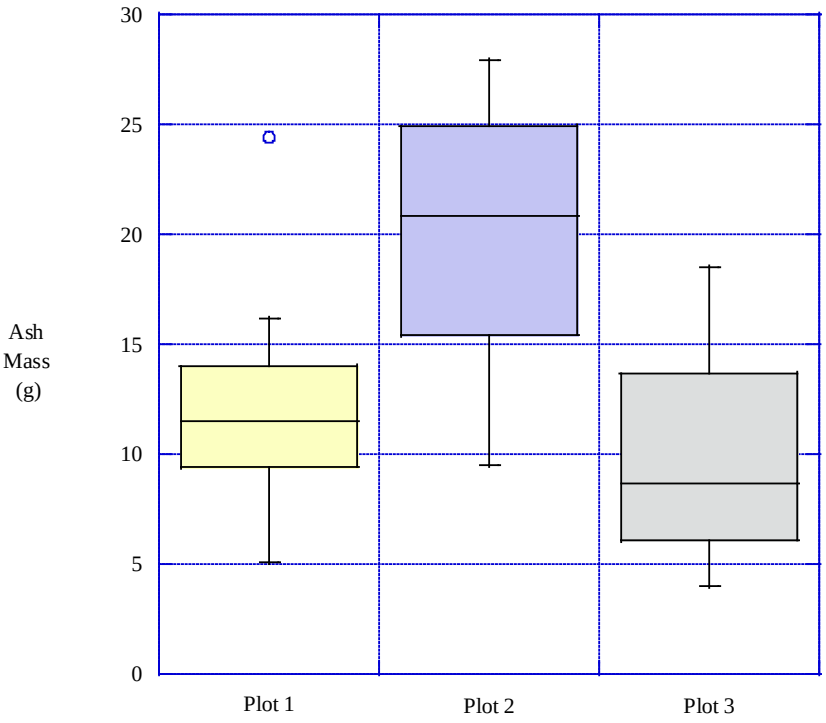


Figure 7-4. Distribution of Biosolids for Each Land Sampling Plot Based on Ash Mass (Data are displayed using boxplots. Plots 1 and 3 were statistically similar, 10.8 ± 4.4 g ash mass of biosolids/ 900 cm^2 geotextile, and showed an even distribution of biosolids across the plot. Plot 2 had a statistically higher level of biosolids ash mass, 20.0 ± 5.6 g ash mass of biosolids/ 900 cm^2 geotextile, and an uneven distribution of biosolids across the plot.)

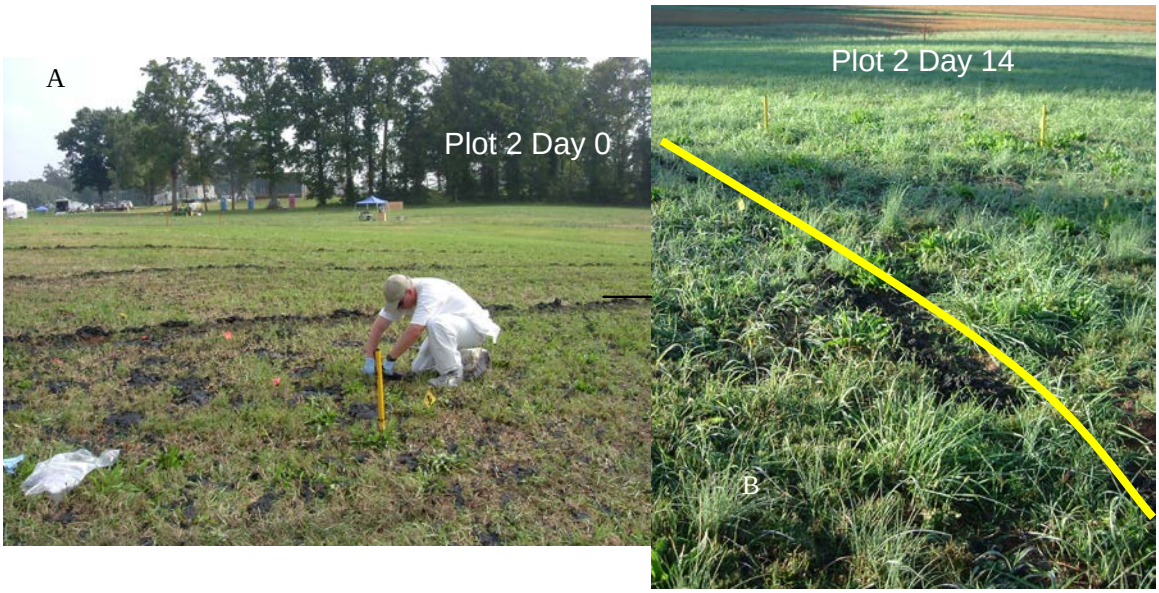


Figure 7-5. Photographs of Land Application of Biosolids on Plot 2 (In A, the banding pattern on the field from the spreader crosses the photograph. In B, Plot 2 is shown on Day 14; the band of biosolids runs below the yellow line.)

Using all the data from the geotextile squares, the application rate was 7.3 to 9.5 wet tons/acre or 1.7 to 2.2 dry tons/acre (95% confidence interval). This rate closely approximated the planned rate of 10 wet tons/acre.

Based on a review of the peer-reviewed literature, biosolids land application studies have not characterized biosolids distribution. In general, dry weight application rates were reported. In a few cases, large tarps were used to measure application rates. In this study, most samples were collected in discrete soil cores within specific plots. In addition, statistical analysis identified plot-specific observations for several analytes. Therefore, global estimates of biosolids application are not as informative as the data produced by the technique used in this study. The technique used to measure the amount and distribution of applied biosolids worked well for this study. Quantities were documented, and data and statistical analysis supported qualitative observations about distribution. Based on the experience at this site, similar data collection efforts are recommended for other studies.

7.4.4 PLFA. Phospholipid fatty acid (PLFA) analysis is a useful measurement to characterize the total microbial community inhabiting a soil sample. This measurement involves extracting a soil sample and isolating the phospholipids that comprise the cell membranes of microbes. Microbes produce different fatty acid molecules in their cell membranes based on the type of microbe and in response to environmental conditions. PLFA does not rely on culturing the cells; a snapshot of the community present is obtained rather than an estimate based on culturable organisms. Total biomass is measured using the quantity of lipid phosphate extracted from a sample, while community structure is characterized by the relative abundance of individual phospholipid fatty acids in a sample (Vestal and White, 1989). This technique is powerful for community level insight, but since pathogens comprise a relatively small fraction of the microbial biomass, PLFA does not provide insight into pathogen levels.

For this study, the objective of PLFA biomass and community structure data collection was to characterize microbial conditions during this study. PLFA biomass data were collected at three locations and three depths from each plot during each sample event. ANOVA was used to evaluate the effect of

plot, sample time, and sample depth as well as two-way interactions. The p-values of statistically significant factors are shown in Table 7-3. Further analysis revealed that plot differences were significant in surface samples, and, therefore, discussions of plot and time differences are focused in this horizon.

Table 7-3. Total Biomass ANOVA Results for Statistically Significant Factors

Factor	P-value
Plot	0.0062
Time	<0.001
Depth	<0.001
Time by depth	0.0012

The total biomass in surface samples for each plot is shown in Figure 7-6 before and after biosolids application. Initially, total biomass varied somewhat among the plots, but this variation was not significant compared to the variation within each plot. Biosolids application increased total biomass by 60 nmol/g dry weight (gdw) on average. This change may have been due to added organisms as well as growth of indigenous microbes. Unfortunately, due to variation within plots, the difference between pre- and post-application biomass could not be statistically separated for the plot pairs.

The surface (0 to 5 cm) total biomass data are graphed as a function of time in Figure 7-7. The mean total biomass for the samples at 10 to 15 and 20 to 25 cm depths are also shown. Statistical analysis of these data revealed that total biomass data decreased with depth. The decrease in biomass as a function of soil depth has been shown with many different soils (Vestal and White, 1989). The subsurface samples were consistent among the three plots and did not change with time, and, therefore, data from all time events and plots were used to compute the 10 to 15 cm mean and error bars depicted in Figure 7-7. The 20 to 25 cm graphical depiction was computed in a similar fashion. The surface samples demonstrated time-dependent behavior. Following biosolids application, Day 14 values were 100% higher than the pre-application levels. Unfortunately, sample-to-sample variation was substantial in these samples, often around 30%. Thus, it is not possible to state that the total biomass changed in the post-application period.

Community structure data were evaluated using PCA and HCA. PCA representations are shown in Figure 7-8A for the surficial samples (0 to 5 cm) and in Figures 7-8B and 7-8C for the 10 to 15 cm and 20 to 25 cm depths, respectively. The axes in each graph were determined by the statistical algorithms of PCA and each dataset. In general, axes are a weighted linear combination of several measurements for each sample. The distance between points indicates differences. The numbers next to each point in the three graphs correspond to the sample event. Based on PCA, the surficial microbial community broke into three groups: biosolids, samples from immediately after application (Days 0 and 14), and pre-application samples clustered with Day 28 and later samples. Within the pre-application and more than 28 days post-application cluster, Day -3 samples were shifted to one side. This shift was the result of a rain event earlier that day. The rain caused a change in fatty acids related to cell dormancy (18:1w7c, 16:1w7c, cy17:0, and cy19:0). This break in dormancy, increased proportions of 18:1w7c and 16:1w7c with decreased proportions of cy17:0 and cy19:0, could have been a function of decreased osmotic stress and/or end of starvation/cellular growth (Kaur *et al*., 2005). Overall, the plot of surface PLFA data suggested that the microbial community present on the soil surface was changed by biosolids application, but the community returned to the pre-application state by the Day 28 sample event.

The PCA of the microbial community structure for the 10 to 15 cm depth showed a clustering of the Day -3 samples (Figure 7-8B). The microbial fatty acids associated with dormancy had shifted as in

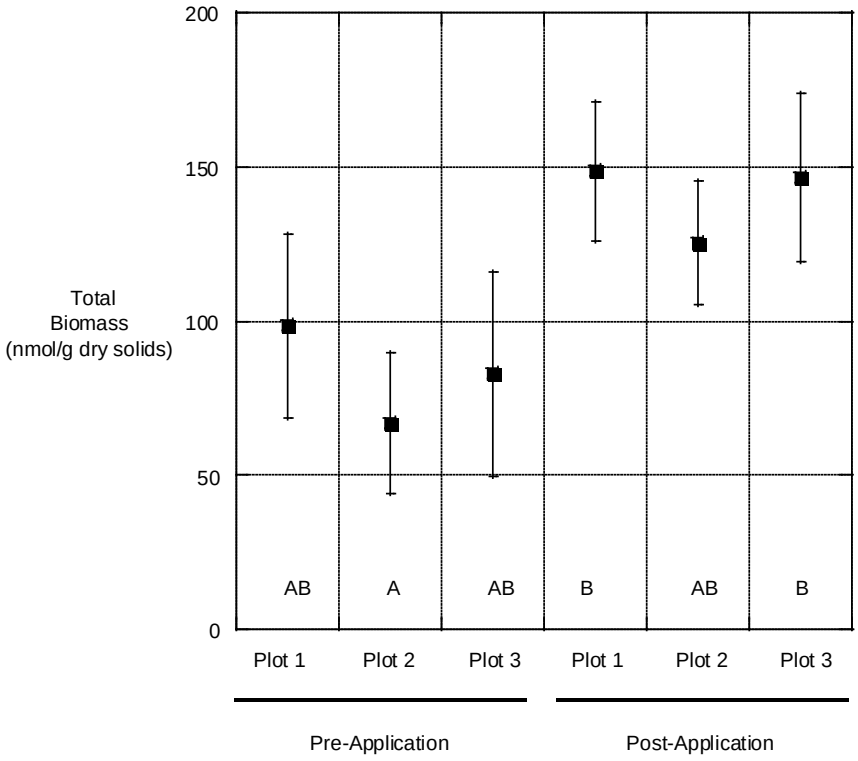


Figure 7-6. Total Biomass for Surficial Samples, 0-5 cm, in Each Plot Before and After Biosolids Application (The means are plotted, and the error bars are twice the standard error. The letter below each box indicates the statistical grouping based on ANOVA. Symbols that share a letter are statistically similar. For example, all pre-application samples share an A designation and are similar. Pre-application Plot 2 and post-application Plot 1 are designated A and B, respectively, and have statistically distinct levels of total biomass.)

the surface samples. As noted in the field observation book, on Day -3 (September 27, 2004), samples were collected during a light rain/drizzle and the soil was evenly moist down to 25 cm. The rest of the samples did not display any discernable pattern, and, therefore, the biosolid application did not appear to affect the soil microbial population at this depth for the length of this study.

The PCA of the microbial community structure from the 20 to 25 cm depth showed no discernable pattern for all soil samples taken (Figure 7-8C). At this depth, the microbial community remained stable for the length of the study.

A review of the peer-reviewed literature identified one relevant study (Peacock *et al*., 2001). A field was amended with manure and ammonium nitrate. The organic (manure) amendment only influenced the microbial community structure in the 0 to 5 cm depth, similar to the observations in this report. However, the literature study differed in that field treatments had been applied six times over 5 years, and the conclusion was based on a single sampling event after treatment. The long-term manure applications changed the soil structure and, therefore, changed the microbial community structure. The single application evaluated in the reported study did not result in a long-term change in the microbial community structure.

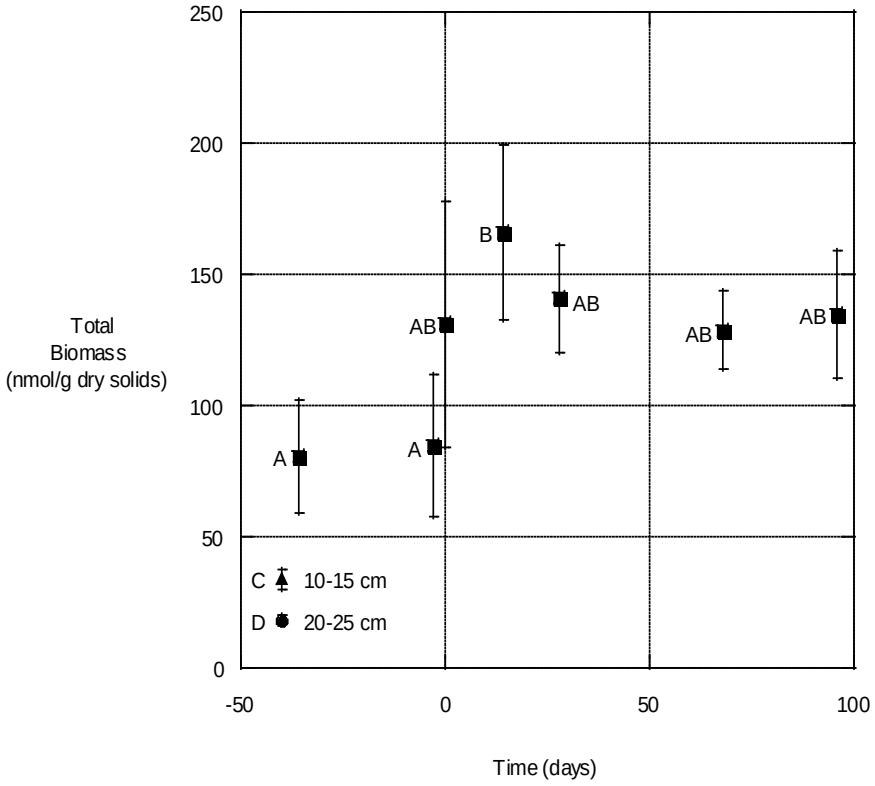
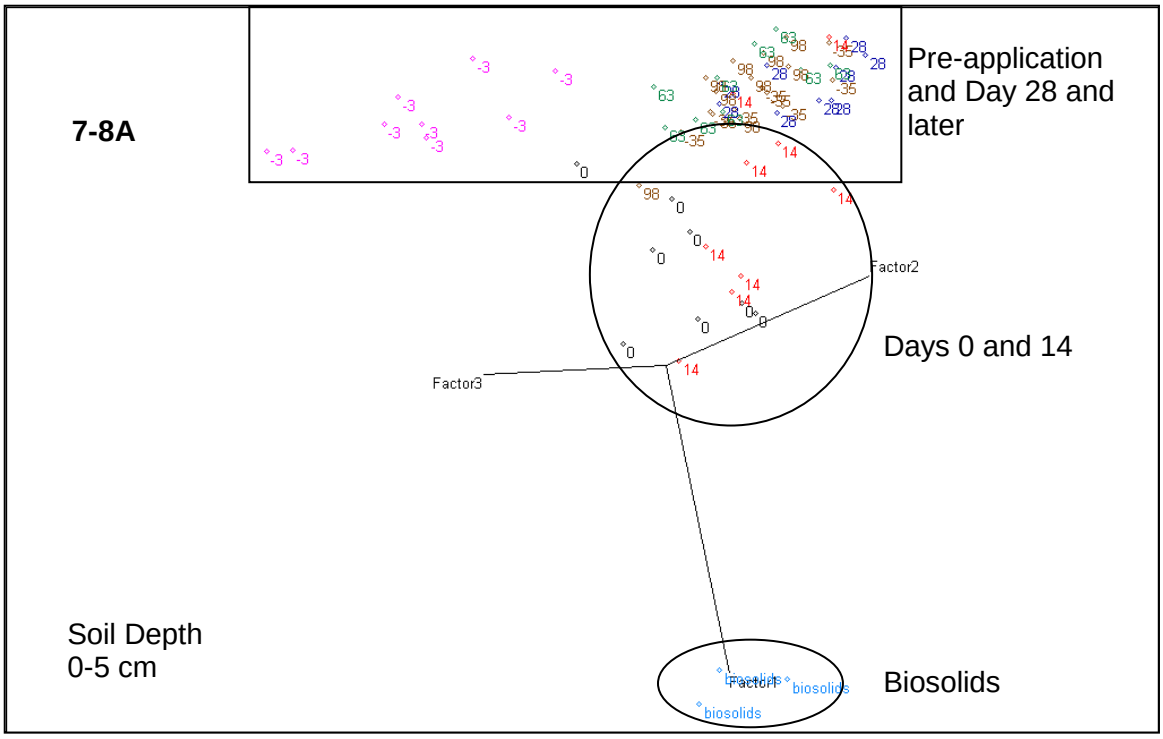
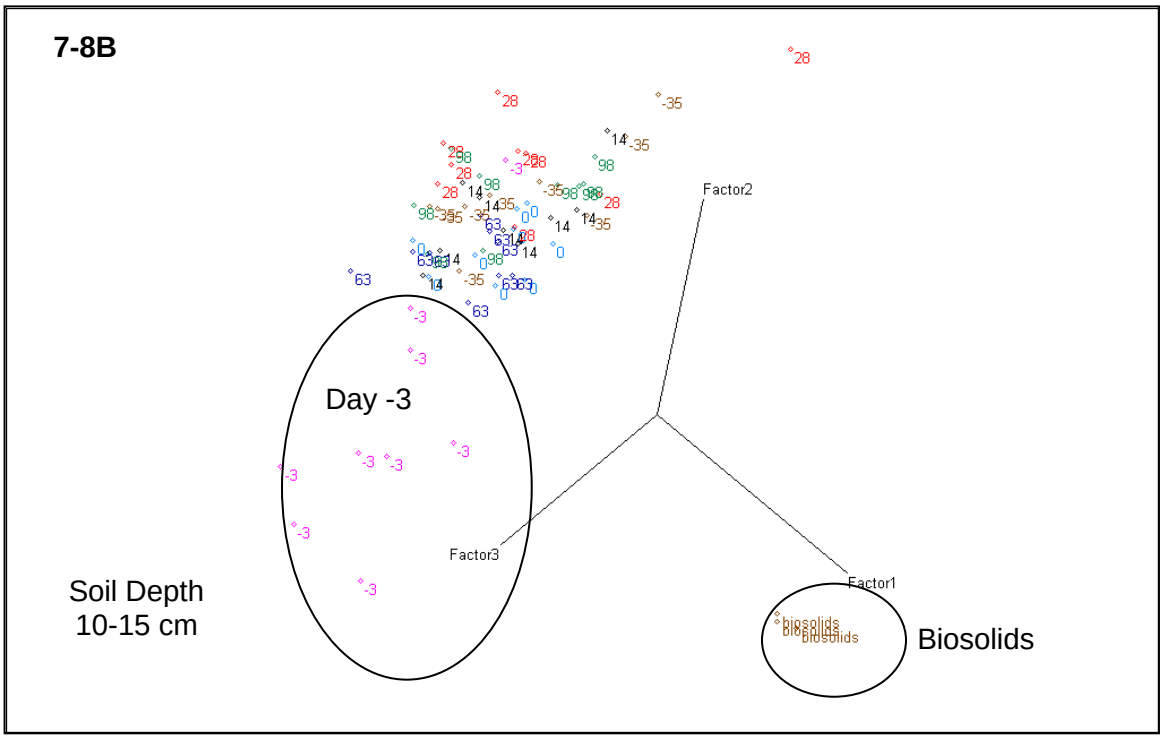


Figure 7-7. Total Biomass as a Function of Time After Biosolids Application for Surficial Samples, 0-5 cm (The means are plotted, and error bars show twice the standard error. Samples at deeper depths, 10-15 and 20-25 cm, are also shown. Since statistical analysis showed no significant temporal variation, graphed values were based on all data at that depth over the study. The letter near each symbol indicates the statistical grouping based on ANOVA. Symbols that share a letter are statistically similar. For example, the surficial data at Days -35 and -3 share an A designation and are statistically similar. The Day 14 level is denoted B and is statistically different from Days -35 and -3 data.)

Based on the experience of this study, PLFA measurements documented short-term changes in the community structure during the sampling period. PLFA-based community structure assessments displayed changes correlating to changes in soil conditions, such as the rainfall immediately prior to biosolids application. In addition, changes were observed following biosolids application. The microbial community returned to pre-application structure after 28 days (see Figure 7-8A).

PLFA-based biomass measurements document differences with soil depth (see Figure 7-7). Data indicate there may be differences based on sample time and plot, but these differences were not statistically significant. In future studies, more replicates of shallow samples are recommended. In addition, it would be useful to collect PLFA-based biomass measurements for a longer period after application. If budgets limit sample collection and analysis, sample collection should be focused on the shallow soil horizon as this depth was the most dynamic.


7-8A
Pre-application and Day 28 and later
Days 0 and 14
Factor2
Factor3
Soil Depth
0-5 cm
biosolids
Biosolids


7-8B
Day -3
Factor2
Factor3
Factor1
Soil Depth
10-15 cm
biosolids
Biosolids

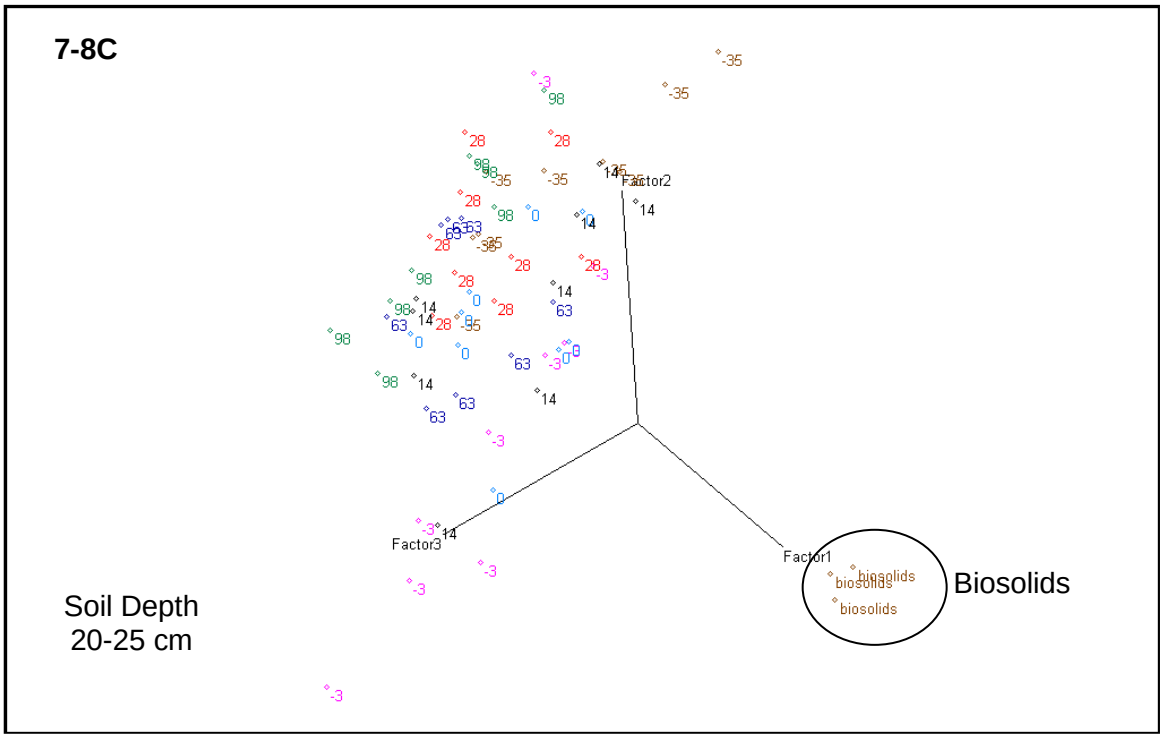


Figure 7-8. Community Structure Based on PLFA Profile: A. 0-5 cm Depth; B. 10-15 cm Depth; and C. 20-25 cm Depth (Numbers next to each point correspond to sample event.)

7.4.5 Fecal Coliforms. Fecal coliform data were collected during this study as an indication of pathogen bacterial behavior following land application of biosolids. The fecal coliform samples were taken from the three different plots at seven different time points (two pre-application, one on the day of application, and four post application). The samples were sent to Environmental Associates, Inc. for analysis. Sample values were reported, but data quality information was not. Results are tabulated in Appendix D (Table D-2). The laboratory reported semi-quantitative values for 48% of the samples (values of less than or greater than a specific number). The QAPP did not anticipate this semi-quantitative data in the statistical analysis plan. To facilitate statistical analysis of the data, censored values were assigned the value of the boundary number for that sample. For example, at Day -36, one sample density was reported as <0.22 MPN/gdw. This sample was assigned a value of 0.22 MPN/gdw for statistical purposes. In another example, one of the Day 63 samples was observed to have a density of $>2.19 \times 10^4$ MPN/gdw. For statistical analysis, this sample was assigned a value of 2.19×10^4 MPN/gdw. Figure 7-9 displays the data using this convention for all three plots. Censored values are noted as open symbols, while quantitative data are shown as closed symbols.

Statistical analysis of these data involved several steps. The distribution of the fecal coliform densities was extremely right-skewed, and, thus, natural logarithm transformation of the density was used so that the ANOVA assumptions could be met. ANOVA indicates that time and time-by-plot interaction were both statistically significant in the model, with p–values of less than 0.0001 and 0.0203, respectively. Therefore, the temporal trend in densities was different for each plot. Because of the significant interaction effect, multiple comparisons were performed within each plot to examine

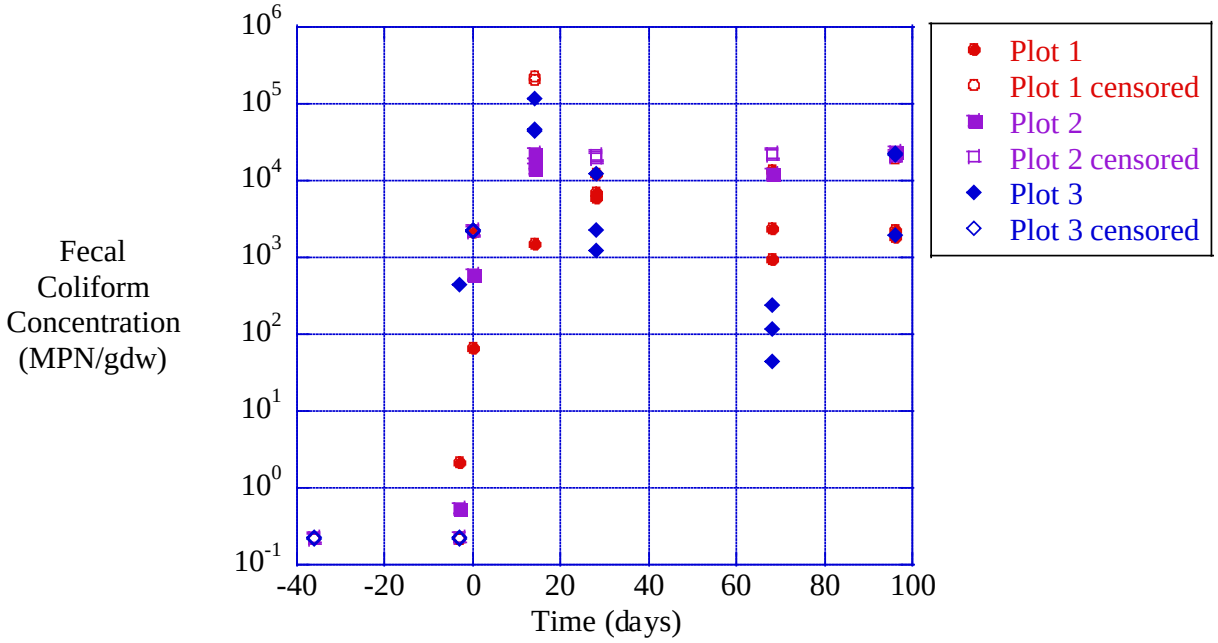


Figure 7-9. Fecal Coliform Concentrations as a Function of Time and Plot

differences in fecal coliform densities over time. Scheffé's method was used to provide a set of confidence intervals with joint 95% confidence. No differences in fecal coliform densities were present between the two pre-application times for any plot. For all three plots, statistically significant differences were identified between pre-application and post-application sample events, with higher estimated densities for the post-application events. In the 28 days after application, differences were observed in the plots. For Plots 1 and 2, the fecal coliform concentrations increased following application and remained higher throughout the post-application period. For Plot 3, the fecal coliform concentration increased after application, reached a maximum on Day 14, and remained elevated through Day 96. For all plots, the densities are stable from Day 28 to the end of the study, but higher than the pre-application levels. The soil temperature began dropping after Day 28, which may have contributed to this plateau in fecal coliform densities.

Fecal coliform measurements exhibited a statistically significant increase following land application of biosolids of more than 100-fold. Post-application levels were stable from Day 28 through the end of the sample period at Day 98. Differences were observed between plots. Since data quality information was not available and analysis included censored data, uncertainty in these conclusions was substantial. Follow-on studies, including the use of replicate plots for fecal coliform sampling are recommended. In addition, more sample replicates within a plot, more replicate plots, and more precise data from the analytical laboratory may enable discrimination of temporal changes and those changes due to differences in biosolids application. In addition, extending the sampling period following biosolids application may be useful.

7.4.6 Alkylphenol Ethoxylates. The APE soil concentration was measured as a function of time to better understand the persistence of these chemicals following biosolids land application. APE samples were collected at three depths in each plot at each sample event. They were not observed in any sample.

The concentration of all analytes was below the detection limit in all pre-application samples. Since the field had no prior exposure to biosolids, pre-application concentrations of NP and OP were set at zero for statistical purposes. The APE degradation products OP and NP were observed after biosolids application. NPs were observed in shallow samples but not consistently at lower depths. OPs exhibited similar behavior. Thus, the statistical analysis was limited to surficial data. After biosolids application, concentrations reported as below detection limit were assigned the reporting limit for statistical purposes. The NP data are graphed in Figure 7-10 as a function of time and plot.

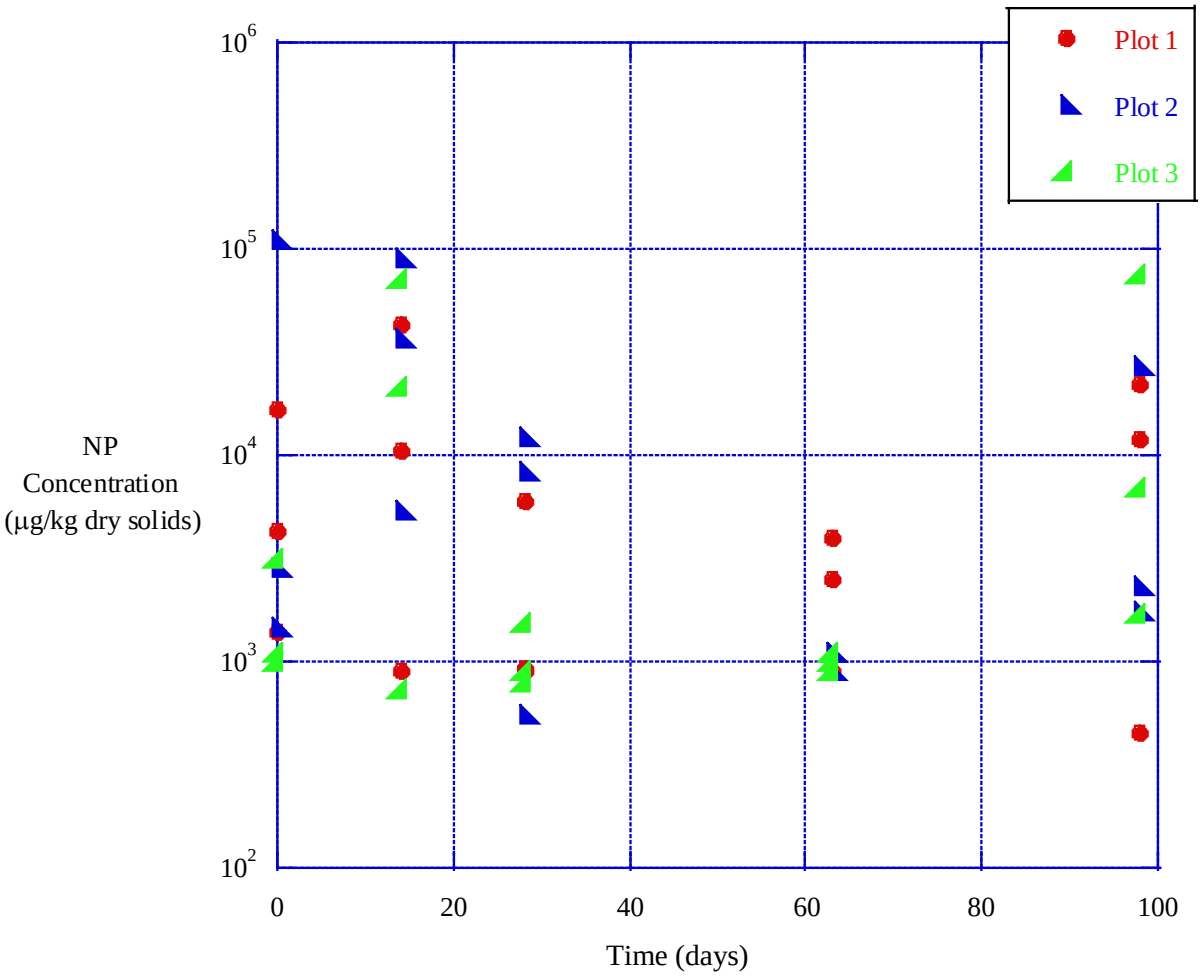


Figure 7-10. NP Concentrations as a function of Time and Plot in Surficial Samples, 0-5 cm, after Biosolids Application (Concentrations of each replicate are graphed. Prior to biosolids application, samples did not contain detectable levels of NP. For the purposes of statistical analysis, these pre-application samples were set at zero.)

Data quality measures for APEs were within acceptance criteria with one exception, surrogate recoveries. Surrogate recoveries were lower than acceptance criteria in 26% of the samples. However, specific acceptance criteria were not established for this soil. Lower surrogate recoveries are consistent

with higher clay contents, and this soil had significant clay content. Thus, reported concentrations may be biased low but were useful for understanding NP/OP behavior in this setting.

A MANOVA model was used to examine the effects of the two factors (plot and date) on NP and OP concentrations. This analysis revealed that the date-by-plot interaction was not statistically significant, so it was dropped from the model, leaving only the main effects of date and plot. Wilks' Lambda test indicated that date was a significant predictor of the joint NP/OP responses (p-value = 0.0032), but plot effect was not found to be statistically significant, with a p-value of 0.5248. However, even though the multivariate Wilks' Lambda test indicated that the date was statistically significant in jointly predicting NP/OP concentrations, multiple comparison could not discriminate differences among the seven dates for NP and OP concentrations. In other words, the statistical analysis did not identify any changes in concentration.

Other researchers have also considered the fate of NP in biosolids-soil systems. When anaerobic biosolids are combined with soils to form homogeneous mixtures, removal can be quick: 80 to 2.4 mg/kg dry soil in 30 days (97% removal, Hseu, 2006), and 0.246 to 0.064 mg/kg dry soil in 30 days (74 % removal, Mortensen and Kure, 2003). However, two studies report slower removal: NP mineralization of 58 % in 38 days (Hesselsoe *et al*., 2001), and 10 % in 150 days (Dettenmaier and Doucette, 2007). Two field studies have shown very disparate results. In one, anaerobically digested sludge was applied as a liquid to a grass field at 6 dry tons/acre and monitored for 320 days (Marcomini *et al*., 1989). Initial removal rates were high; concentrations decreased from 4.7 mg/kg dry soil to about 1 mg/kg in 3 weeks. At that point, the rate slowed, and a plateau concentration persisted at 0.5 mg/kg dry soil (89% removal) through the remainder of the study. When anaerobically digested biosolids were applied as a wet cake at 8 dry tons/acre, NP was not detected 31 days after application, but after 156 days, NP was measured at 3.6 ± 0.8 mg/kg dry soil (mean $\pm$ standard deviation, Kinney *et al*., 2008). The results for the second field study are similar to the results for this study, persistent and variable observations of NP following land application of biosolids.

In a series of studies, Hesselsoe *et al*. (2001) observed that NP degradation was fastest in homogeneous aerobic mixtures of soil and biosolids and degradation was retarded in non-homogeneous mixtures containing biosolids aggregates. NP mineralization in 4-cm aggregates was 51% of the mineralization observed in 2-cm aggregates after 119 days. Oxygen penetration into these aggregates is slow, and the aerobic volume of an aggregate correlated to NP mineralization. These observations were consistent with available field data. Residual concentrations were lower for liquid application where aggregates should be small (Marcomini *et al*., 1989) than for cake application (Kinney *et al*., 2008). Biosolids particle size was not measured in this study, but as shown in Figure 7-3B, the biosolids did not form a fine granular material when applied. Rather, they formed clumps of varying size and thickness. This physical distribution and the high moisture content would limit oxygen penetration into this material and, thus, NP removal.

Nine replicates at each sample point were not sufficient to statistically distinguish concentration changes with time after application; however, this dataset can be used to evaluate the effect of additional samples on statistical power ($1 - \beta$). Van Belle and Martin (1993) developed expressions relating statistical power to sample replication, variability, and difference between samples. Using the equations for log normally distributed data, $Z_{1-\beta}$ was calculated for sample sizes ranging from 3 to 54 replicates using the summary data from the five sample events. In this calculation, relative standard deviation (σ/μ, RSD) was assumed to be equal for both samples and the ratio of means (μ_1/μ_2 or f) was set at 1.25. An Excel spreadsheet was used to compute $1 - \beta$ at $\alpha = 0.05$. Results from these calculations for the lowest and highest RSD observed in this study are presented in Figure 7-11. Power increases with sample replication. For the case with the lowest RSD, power does not exceed 60% with more than 50 samples

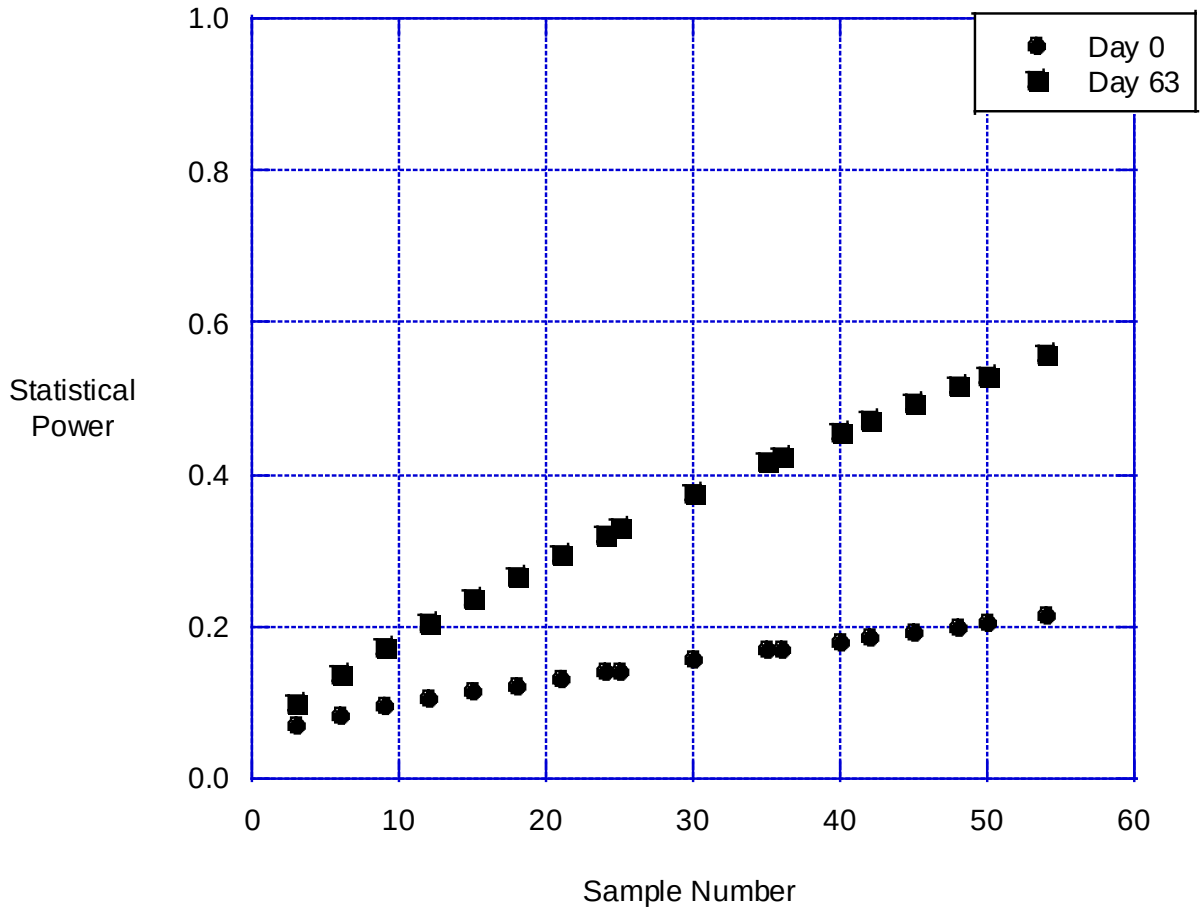


Figure 7-11. Statistical Power for NP Samples as a Function of Sample Number for Day 0 and Day 63 (These sample events had the highest and lowest RSD, respectively. The ratio of means, f, was set at 1.25, and the relative standard deviation was equal. Lognormal calculations were used.)

while the higher RSD barely exceeds 20%. This level of replication would be too expensive for many research projects, and these power levels are too low. This analysis suggests that a different sampling approach is needed. Sampling options to consider include larger samples with homogenization, sample or extract composites, or normalization with a biosolids marker. Field studies are needed to select an acceptable method.

APEs were not observed during the sampling period. Their metabolites, OP and NP, were observed consistently in post-application shallow samples but not in deeper samples. Since no plot effects were observed, conclusions were based on nine replicates. Concentrations increased following application, but variability was too high to identify temporal changes. Based on the experience in this study, a different approach to sampling may be useful in characterizing the persistence of compounds such as APEs. A longer sample period is also recommended. In addition, future data collection to evaluate the persistence of endocrine disrupting compounds (EDCs) should include a broader spectrum of chemicals.

7.4.7 Ecotoxicity Screening. Soil toxicity was screened to evaluate whether biosolids application enhanced or degraded the soil environment. Several assay characteristics were considered in the process of selecting bioassays including test organism, exposure period, and assay endpoint. In general, it is preferable for assays to cover a range of species, exposure periods, and assay endpoints. For example, the assays selected for this study included ecologically relevant plants and animals as test organisms. Also, assays may address a variety of endpoints such as mortality, mutagenicity, growth, or endocrine system disruption. For the assays selected, the endpoints included mortality and growth.

For this study, 14-day earthworm mortality and 5-day seed germination and root elongation assays were used to screen for soil toxicity or enhancement. These assays were generally selected based on their relevance to soil toxicity (Chang *et al*., 1997; Dorn *et al*., 1998; Environment Canada, 1994; Hund and Traunsurger, 1994; Meier *et al*., 1997; Salintro *et al*., 1997; Simini *et al*., 1995; EPA, 1988, 1989). The list was limited and would be strengthened by the addition of chronic sublethal animal assays such as earthworm growth and reproduction, earthworm avoidance, or longer exposure plant assays. However, the current assay list provides some toxicological information; testing costs and development time prevented using a larger collection of assays.

Soil samples for ecotoxicity measurements were collected before (Day -35) and after land application (Days 0 and 98) and were transported to the laboratory for testing. In the laboratory, sample soil was mixed with artificial soil to produce five different concentrations of test soil. Then, organisms were placed in the soil mixtures and incubated for 5 days (plants) or 14 days (earthworms). The response to the concentrations of the test soils was measured.

The toxicity tests for these samples followed the QAPP with one exception, oat seed age. The seeds used in the oat seed germination and root elongation assays were taken from a shipment of oat seeds received in 2002. This seed holding time exceeds QAPP specifications. However, since subsequent shipments have not met QAPP accuracy requirements for root elongation in controls and the 2002 seeds continue to meet these requirements, the 2002 seeds were used.

Prior to performing ecotoxicity tests, various soil parameters, including soil pH, were evaluated so that the tests could be conducted in a consistent fashion. For all but one soil sample from this study, pH was low, ranging from 4.7 to 5.7. The QAPP specified that if soil pH was below 6.0, calcium carbonate ($CaCO_3$) would be added to raise the pH to at least 6.5. Preliminary testing showed that 1-mass percent $CaCO_3$ raised the pH to an acceptable level. Thus, all samples were amended with 1-mass percent $CaCO_3$ to avoid any question about inconsistent dilution. One sample was tested to determine if the $CaCO_3$ amendment affected the results; no difference was observed.

Ecotoxicity assays met data quality objectives with one exception. The reference toxicant controls for the Day 98 lettuce test did not display the required root reduction. This test was repeated, and the reference toxicant controls met data quality objectives. The retest data were used for statistical analysis.

No earthworm mortality was observed in any sample or for any soil concentration. No consistent changes in seed germination effects were observed. Thus, these assays were not sensitive to any changes associated with land application of biosolids at this site and under these conditions.

Root elongation data for lettuce and oats demonstrated changes. The data consisted of three replicates from each of three plots at three time points and five different test soil concentrations. Data are graphed in Figure 7-12. In the presence of a toxic soil, roots are usually longer in the 0% sample soil and decrease as sample soil concentration increases. In this study, the root length data for both lettuce and oats did not display the typical pattern as a function of soil concentration. The 0 and 100% soil concentrations showed longer roots than the intermediate concentrations. The cause of this unusual

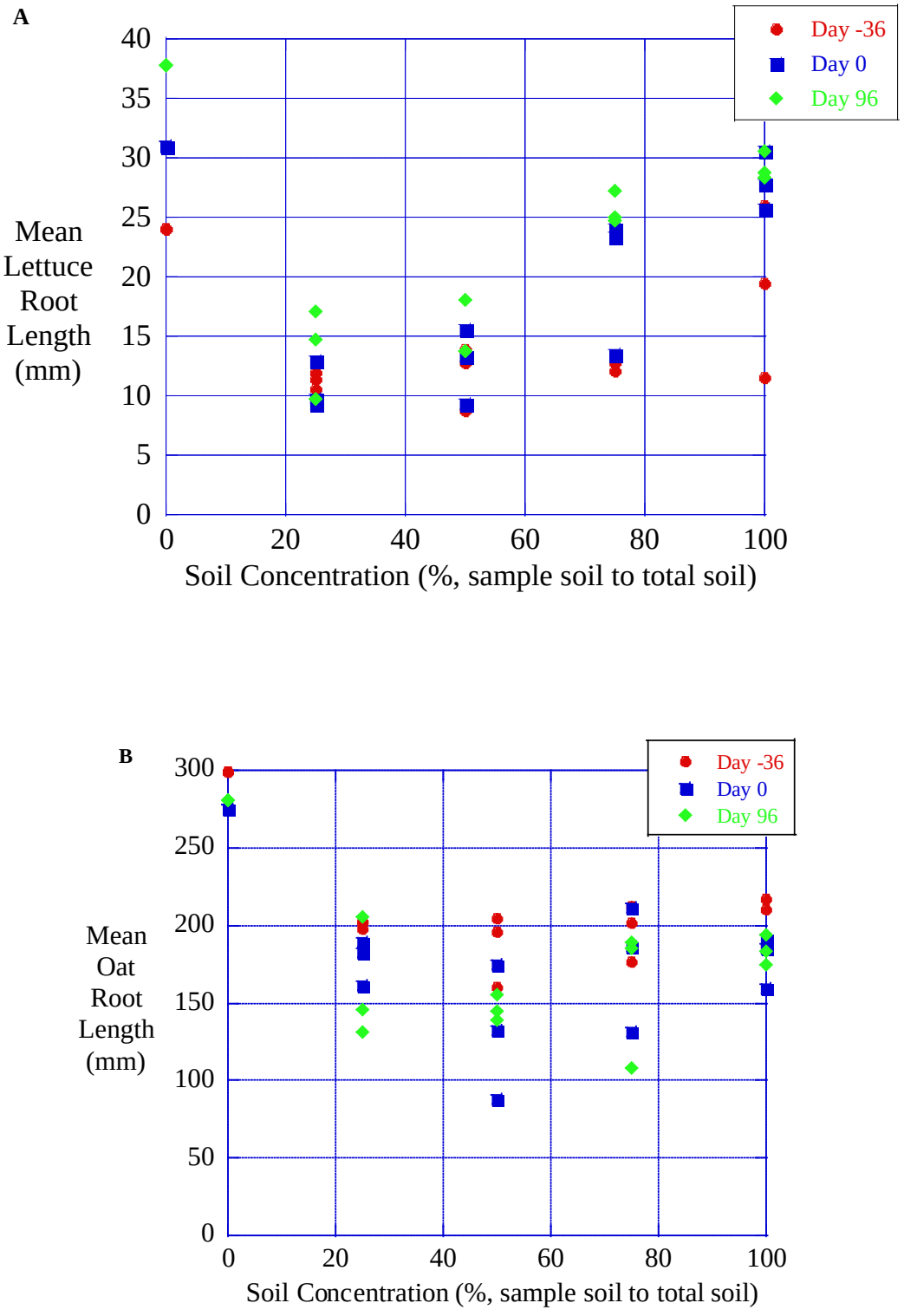


Figure 7-12. Root Elongation as a Function of Test Soil Concentration and Sample Event: A – Lettuce Data; and B – Oat Data

behavior is not known. The characteristics of the sample soil and artificial soil were quite different. It was possible that varying soil texture and nutrient levels may have played a role in these observations. This unusual pattern was observed in all samples, before and after biosolids application.

Data were evaluated using a three-way ANOVA for the two species. Results are shown in Table 7-4. Root lengths did not differ between plots. Differences were observed in response to sample time and sample concentration for both lettuce and oat roots. For lettuce, the time-by-concentration interaction was also statistically significant, which indicates that the effect of sample time on root lengths differed for the five sample concentrations. Statistical analysis of the concentration dependent data was used to maximize knowledge gained from the study. However, the focus of this data collection effort was to understand the effect of biosolids application on soil organisms, and, thus, the time dependent changes are of greater interest.

Table 7-4. Three-Way ANOVA Results for Root Elongation

Effect	Lettuce p-value	Oats p-value
Plot	0.8825	0.7237
Time	<.0001	0.0004
Concentration	<.0001	<.0001
Time-by-Concentration	0.0121	

For lettuce, root lengths increased over the course of the study. In Figure 7-12A, the Day 98 samples appear consistently longer than for the other days. Statistical analysis indicates that each time point was distinct, as illustrated in Figure 7-13, and root lengths for lettuce increased with time, both from pre-application day to the application day and from the application day to the final sample. This observation supports the use of biosolids as a beneficial soil amendment.

The observations for oat roots were different in that the roots decreased in length throughout the course of the study. The root lengths for the final sample event appear to be generally lower than for the other events in Figure 7-12B. The statistical analysis confirmed this observation. Oat root lengths decreased with collection time. The decrease was statistically significant between the pre-application and application days, but there was no statistically significant change between the application day and the final sample. This dataset does not support the use of biosolids as a beneficial soil amendment.

Ecotoxicity following use of biosolids as a soil amendment was evaluated by Banks *et al.* (2006). They evaluated anaerobically digested biosolids from 19 locations where biosolids are typically land applied. Samples of biosolids and soils to which biosolids had been applied were tested. Earthworm tests included a 14-day biomass assay and a 7-week assay measuring biomass, numbers of juvenile hatchlings, and cocoon numbers and hatchability. Plant assays included a 5-day seed germination assay using lettuce, radish, and millet and a 10-day root elongation test using lettuce. The paper describes slightly different 5-day seed germination assay methods than were used in this study. In Phase I, biosolids were added to soil at one concentration. Lettuce germination was rarely affected, but earthworm biomass accumulation was reduced in six of the 19 biosolids samples. Five samples from Phase I were evaluated further in Phase II. Lettuce germination was significantly reduced in one sample, possibly due to elevated salinity and low pH in that sample. Radish and millet exhibited similar differences. Lettuce root lengths were shorter in biosolids amended soils, but the difference was not statistically significant. Earthworm biomass gain was lower than controls in two samples and similar to controls in three samples. Both

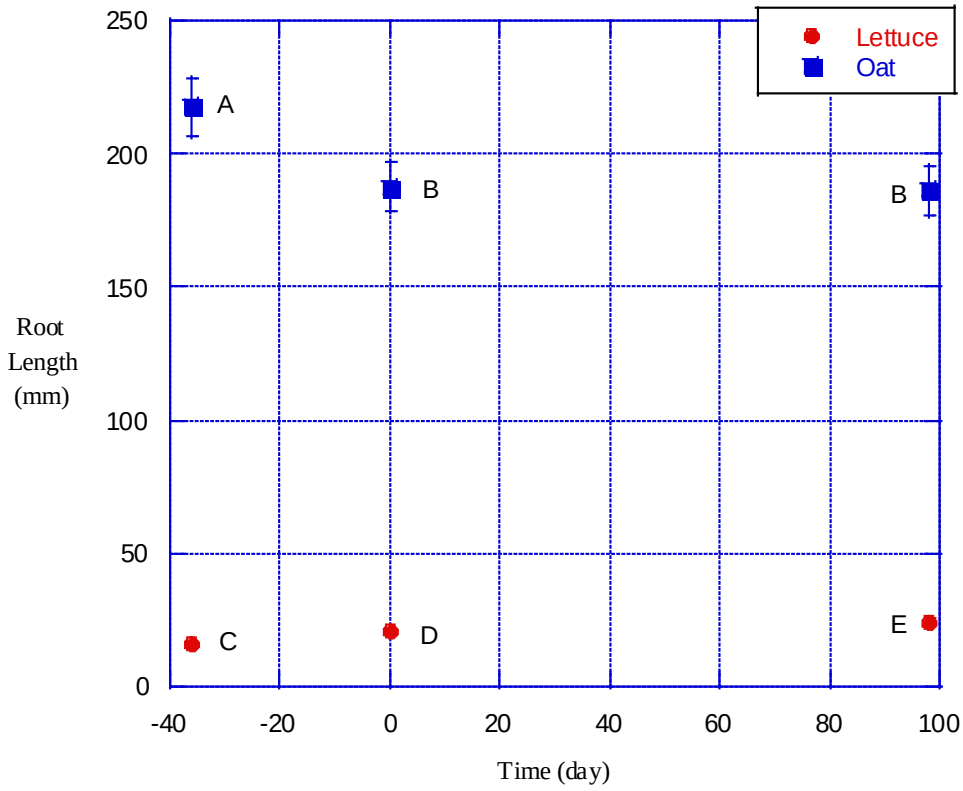


Figure 7-13. Average Root Length for all Soil Concentrations as a Function of Treatment Time for Lettuce and Oats (The 95% confidence limits are indicated by the error bars. The letter near each symbol indicates the statistical grouping based on ANOVA. Symbols that share a letter are statistically similar.)

samples with lower biomass gain displayed higher ammonia levels; one sample also had elevated saline and low pH. In summary, Banks *et al*. (2006) used a more varied selection of bioassays to evaluate the ecotoxicity of biosolids application to land. Based on six bioassays measuring 10 endpoints, 19 of 110 toxicity tests showed a negative effect, but no consistent trends were observed in biosolids applied in a manner consistent with 503 Regulations.

Therefore, based on the literature, screening ecotoxicity results did not exhibit a consistent pattern. In this study, no change was observed in earthworm mortality and seed germination data following biosolids application. Since both assays displayed a maximal response prior to application, no added benefit of biosolids could be demonstrated. Neither assay showed a negative response to biosolids.

Lettuce root length displayed enhanced growth following biosolids application in this study. However, oat root length showed reduced growth. Based on this information, continued toxicity screening is recommended for future studies. In addition to the current assays, chronic earthworm tests with non-lethal endpoints, such as biomass accumulation, and longer-term plant studies would be useful in gaining a fuller understanding of the effect of biosolids. If a particular organism is of interest, assays directed toward that organism are recommended. A longer sampling period may be useful.

7.4.8 Other Microbial Measurements. The microbial community was evaluated using several techniques for this study. PLFA/FAME measurements characterized the size and diversity of the

community. Fecal coliforms were measured as a potential indicator of pathogenic bacteria. In addition, other microbial indicators were measured including VHO, *Salmonella*, enteric viruses, coliphage, total heterotrophs, fecal coliforms, and *Enterococcus* spp. Due to the number of tests and costs of analysis, this sampling was conducted with less replication than the replication used for the fecal coliform analysis discussed earlier. One composite sample was collected from each plot during each sample event (see Table 7-1).

Most samples were sent to Environmental Associates, Inc. for analysis. Sample results were reported, but data quality information was not. Results from this data analysis are shown in Table 7-5. With the exception of biosolids samples, rare detections were observed for the enteric viruses, *Salmonella*, and VHO following biosolids application. Somatic coliphage detections were more common.

Table 7-5. Other Microbial Indicators from Land Sampling Analyses Performed by Environmental Associates, Inc.

Sample Event	Date Sampled	Plot	Virus[(a)] (MPN/4g)	*Salmonella* (MPN/4g)	VHO (No. viable/4g)	Coliphage	
						Male-specific (PFU)	Somatic (PFU)
Day -35	8/25/2004	1	<0.82	<0.33	<0.56	<1	<1
		2	<0.84	<0.32	<0.47	<1	<1
		3	<0.80	<0.32	<0.48	<1	<1
Day -3	9/27/2004	1	<0.80	<0.32	<0.57	<1	3
		2	<0.67	<0.32	<0.53	<1	16
		3	<0.67	<0.32	<0.60	<1	7
Day 0	9/30/2004	1	<0.67	<0.36	<0.47	<1	1
		2	1.37	<0.35	<0.56	<1	2
		3	0.68	<0.37	<0.68	<1	4
Day 28	10/28/2004	1	<0.67	6.06	<0.44	<1	1
		2	<0.67	1.59	<0.48	<1	<1
		3	<0.68	<0.36	<0.48	<1	<1
Day 63	12/7/2004	1	<0.68	0.694	<0.32	<1	1
		2	<0.68	20.85	0.54	<1	<1
		3	<0.68	1.41	<0.52	<1	1
Day 98	1/4/2005	1	<0.68	<0.38	1.14	<1	1
		2	<0.68	1.22	1.13	<1	1
		3	<0.68	<0.43	<0.67	<1	<1
Day 1	10/1/2004	Biosolids	<0.68	>325	1.78	199	557
			2.94	>325	2.46	133	616
			<0.70	>325	<0.77	75	448

(a) All samples <1 PFU/4g

In this study, fecal coliforms were measured as indicators of pathogenic bacteria such as *Salmonella*. *Salmonella* were detected in a total of six soil samples during the study period between Days 28 and 98. Increased *Salmonella* levels in soil samples during this period may have been due to additions to the soil community from the biosolids or growth of indigenous *Salmonella* on biosolids substrates (Unc *et al*., 2006; Winfield and Groisman, 2003). Fecal coliforms levels, which were higher than pre-application levels and stable during this period of the study, were about three orders of magnitude higher than the *Salmonella* levels observed. Both microbial analytes were temporally steady during this period.

Thus, fecal coliforms were more easily measured than *Salmonella*, and showed a pattern of behavior consistent with the available *Salmonella* data. In this study, fecal coliforms were indicators of *Salmonella* behavior during static periods; no data were available to evaluate whether fecal coliforms were indicators of *Salmonella* behavior during dynamic conditions.

The Sustainable Agricultural Systems Laboratory, USDA, analyzed samples at three time events: Day -35, Day 28, and Day 98. Data were not reported with information concerning holding times, positive or negative controls, or other measures of data quality. Therefore, these data are of unknown quality. The results from the USDA data analysis are shown in Table 7-6.

Table 7-6. Other Microbial Indicators Measured by the USDA Laboratory

Sample	Total Heterotrophs	Fecal Coliforms	Enterococci
Biosolids Samples			
Replicate 1	6.61×10^{10}	1.04×10^{9}	2.75×10^{5}
	6.43×10^{10}	1.08×10^{9}	8.26×10^{5}
Replicate 2	1.51×10^{11}	4.87×10^{9}	9.54×10^{5}
	1.49×10^{11}	5.57×10^{9}	2.07×10^{6}
Replicate 3	4.95×10^{10}	5.49×10^{8}	1.80×10^{5}
	4.32×10^{10}	6.30×10^{8}	6.30×10^{5}
Day 28 Samples			
Plot 1, Replicate 1	1.66×10^{8}	1.79×10^{6}	2.60×10^{4}
Plot 1, Replicate 2	1.58×10^{8}	1.48×10^{6}	2.85×10^{4}
Plot 2, Replicate 1	1.22×10^{8}	8.92×10^{5}	2.88×10^{4}
Plot 2, Replicate 2	1.35×10^{8}	1.00×10^{6}	3.30×10^{4}
Plot 3, Replicate 1	3.63×10^{6}	8.85×10^{4}	1.14×10^{4}
Plot 3, Replicate 2	1.80×10^{7}	1.06×10^{5}	1.28×10^{4}
Day 98 Samples			
Plot 1, Replicate 1	7.65×10^{7}	5.43×10^{5}	1.80×10^{4}
Plot 1, Replicate 2	7.42×10^{7}	6.87×10^{5}	1.29×10^{4}
Plot 2, Replicate 1	6.20×10^{7}	2.48×10^{5}	5.45×10^{3}
Plot 2, Replicate 2	8.97×10^{7}	2.17×10^{5}	6.00×10^{3}
Plot 3, Replicate 1	2.57×10^{7}	2.27×10^{5}	1.76×10^{4}
Plot 3, Replicate 2	1.95×10^{7}	1.56×10^{5}	1.90×10^{4}

Units on all measurements are CFU/g dry solids. Data for Day -35 samples were not reported on a dry basis and, thus, were not included. *S. aureus* and *Salmonella* were measured by spread plating, but all values were below the detection limit.

While both labs measured fecal coliforms and *Salmonella*, it is difficult to compare results. *Salmonella* detections were very infrequent; there are little data to compare. Fecal coliform samples are processed in different ways in the two laboratories, and results (Figure 7-9 and Table 7-6) are reported in different units. Fecal coliforms results from Day 28 and 98 for both labs were shown in Table 7-7. In general, the MPN results were two orders of magnitude lower than the CFU results. However, the change between days within a measurement technique was minimal. This observation was consistent with statistical analysis of the Environmental Associates, Inc. data, i.e., no significant changes were observed between Day 28 and the final sample event at Day 98.

Table 7-7. Fecal Coliform Results from Environmental Associates, Inc. and USDA (Data were presented as a function of plot and time because statistical analysis of Environmental Associates, Inc. data identified statistically significant time-by-plot interactions. Data quality for these analyses were unknown as neither laboratory supplied this information.)

Plot	Results from EAI (MPN/g dry wt)			Results from SASL, USDA (CFU/g dry solid)	
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2
Day 28					
1	7.00×10^3	6.02×10^3	1.23×10^4	1.79×10^6	1.48×10^6
2	$> 2.13 \times 10^4$	$> 1.96 \times 10^4$	$> 2.05 \times 10^4$	8.92×10^5	1.00×10^6
3	2.28×10^3	1.25×10^3	1.21×10^4	8.85×10^4	1.06×10^5
Day 98					
1	$> 1.95 \times 10^4$	2.25×10^3	1.88×10^3	5.43×10^5	6.87×10^5
2	2.38×10^4	$> 2.09 \times 10^4$	$> 2.18 \times 10^4$	2.48×10^5	2.17×10^5
3	2.26×10^4	1.95×10^3	2.21×10^4	2.27×10^5	1.56×10^5

The biosolids used in this study contained many microbial indicators, but detections in soil samples were rare. Since other measurements displayed high variability, future studies should evaluate whether the microbial indicator sample strategy yields representative samples. Otherwise, this sample approach is appropriate and indicates that other microbial analytes were rarely observed following land application of biosolids at this site. A similar approach may be useful in future studies.

7.5 Conclusions

A field-scale research project was conducted in 2004-2005 to evaluate land application of anaerobically digested biosolids at agronomic levels. For this study, biosolids were applied to a fescue field in a 100-m diameter circle by a side discharge manure spreader. Biosolids had not been applied to this land previously. Soil samples were collected from three replicate plots within the application area prior to biosolids application and for 98 days following application. Study conditions were characterized by measuring biosolids mass and distribution after application, PLFA, and agronomic and weather data. Fecal coliform, APEs, total biomass, community structure, and ecotoxicity data were used to evaluate the effects of biosolids land application. Limited data for specific pathogens were collected. *Salmonella* and VHO were observed in biosolids samples but rarely in soil samples.

The measurements used to characterize the study were generally informative. Biosolids distribution for each replicate plot was determined by measuring the dry mass and ash mass of applied material in each plot. Statistical analysis revealed plot-to-plot variations in the amount and distribution of biosolids. This observation supports a recommendation for using replicate plots within a study. In addition, large-scale measures of biosolids application, such as loading rate, may not reflect the biosolids content of discrete samples. Biosolids distribution data documented the wet and dry mass applied per acre. Sampling techniques that enable measurement of biosolids loading and analytes of interest in a single sample may be useful in reducing uncertainties in these types of studies. Soil agronomic data varied primarily with depth and did not exhibit consistent changes following biosolids application. Weather data displayed fairly moist conditions and falling temperatures following application. The soil and weather data collected may be useful when placing this study in context with other similar studies in order to draw general conclusions about land application of biosolids.

Measurements used to evaluate the effect of biosolids application often exhibited changes. However, data interpretation was complicated by semi-quantitative data, sample-to-sample variation, and inconsistent results. PLFA measurements were used to characterize the size and diversity of the microbial community. Total biomass based on PLFA varied from plot to plot, with depth, and with time for surficial samples. Total biomass in shallow samples increased following application, but changes with time after application were not statistically significant. Based on PLFA distribution, the microbial community was shifted following biosolids application, but returned to pre-application structure within 28 days. Fecal coliforms were measured as indicators of pathogenic bacteria. The laboratory reported semi-quantitative fecal coliform data. Statistical analysis identified plot-to-plot variations. An increase in fecal coliform density of more than 100-fold was observed after application that generally persisted for the duration of the study. Therefore, results from total biomass amount and fecal coliform density were consistent, i.e., increased levels following biosolids application and remaining throughout the sampling period. In addition, both exhibited plot-to-plot variations. PLFA community structure results were different, showing a transient change after biosolids application. The transient period in community structure results corresponded to the time period when fecal coliforms and total biomass levels increased. When fecal coliform and total biomass data were stable, before application and from 28 to 98 days after application, community structures were similar. Two characteristics of these measurements should be considered in comparing this information. Both total biomass and fecal coliform data reflect absolute amounts of analyte, while community structure is determined by the relative amounts of PLFAs. In addition, community structure analyses incorporated much more data and, as a result, may be more sensitive to changes in the microbial population than fecal coliform and total biomass measurements.

APEs, including degradation products such as OP and NP, were of interest as potential EDCs and persistent chemicals. Samples were collected at three soil depths. The degradation products were observed in shallow samples following application. Statistical analysis could not discriminate temporal changes in concentration after application. Since these compounds are aerobically degradable in soil biosolids mixtures, the persistence of the chemicals may reflect exposure to anaerobic environments within biosolids particles or microbial preferences for other substrates in biosolids.

Soil toxicity was screened using the 14-day earthworm mortality and 5-day seed germination and root elongation in lettuce and oat bioassays. Soil samples were screened for toxicity prior to, immediately after, and 98 days after biosolids application. These results did not demonstrate a consistent pattern. Earthworm mortality and seed germination were neutral, with no enhancement or reduction following application. Lettuce and oat root elongation showed enhanced and reduced growth, respectively.

Based on all of the measures of biosolids effects, it appeared that soil samples changed following biosolids application. In many cases, these changes persisted throughout the study. In the future, longer sampling periods and improved sampling procedures are recommended to refine observations. In light of the biosolids distribution information, sampling procedures that express data based on the biosolids present in a discrete sample may facilitate data interpretation.

7.6 Discussion and Lessons Learned

Several aspects of the experimental design were useful in describing the conditions throughout the study and evaluating the effects of biosolids application to land. For example, the general experimental design using replicate plots with subsampling of each plot with time was practical. Replicate plots differed based on several analytes. The use of replicate plots facilitated separating plot effects from time or other variables in statistical analysis. Replicate plots are recommended in future studies.

The measurements included in this study were selected to characterize the experiment and evaluate questions regarding biosolids application to land. Within the characterization measurements, biosolids distribution and weather measurements were novel and useful. In particular, measurement of biosolids distribution documented mass applied per acre. PLFA measurements provided characterization data as well as documenting changes with time. Measurements to evaluate biosolids land application included indicators of pathogens, the microbial community, chemicals of emerging interest, and assays to evaluate ecological effects. This range of variables is appropriate to evaluate use of biosolids as a soil amendment. Future studies should include this range of variables and, if possible, expand the measurements within each category. For example, chemicals of emerging interest in future studies could include steroid hormones, fluorotelomer residuals, pharmaceuticals, personal care products, pesticides, brominated diphenyl ethers, atrazine, and vinclozolin. Ecotoxicity testing could also be expanded to include earthworm biomass, longer-term plant assays, and a broader range of test species. Other measures of microbial indicators, such as *Salmonella* or coliphage, could be included to better evaluate residual concentrations following biosolid application. However, as with this study, the availability and cost of analytical methods for these measurements, particularly in a biosolids/soil mixture, may limit inclusion in future studies.

The sampling plan for this study evaluated several variables with time and depth. Changes with time were a question of primary interest. In the cases of total biomass, fecal coliforms, and OP and NP, the concentrations before and after biosolids application were different. Unfortunately, the variability in measurements was high relative to the concentration changes after application. Two recommendations for future studies may improve the ability to draw statistically-based conclusions: 1) evaluate biosolids application over a longer period, and 2) use a different sampling strategy to reduce variability. This study also evaluated whether analytes were transported through the soil. At this site, very little downward migration was observed; however, the site soil contained a high fraction of fine particles (clay) that tend to slow downward migration. Based on these observations, future studies may focus sampling on shallow samples and include fewer samples with depth. This study did not consider whether surface runoff would distribute analytes across the study area or beyond. Future studies may want to incorporate sampling to evaluate the runoff.

Data quality for most measurements was acceptable. Study plans included corrective action for most instances when data quality samples were outside of acceptance levels. Two situations were not considered: 1) semi-quantitative fecal coliform data, and 2) low surrogate recoveries for APEs. The semi-quantitative fecal coliform data limited the ability to draw conclusions on residual concentrations. This variable was of critical interest in this study, and, thus, the data quality gap is a concern. It is recommended that future studies carefully evaluate analytical laboratories to assess whether they can meet study requirements. The APE data had a more minor problem. Surrogate recoveries were low in 26% of samples, possibly due to the high clay content of the soil. Analysis of preliminary samples may have identified the need for site-specific data acceptance criteria for these analytes. Analysis of preliminary samples is recommended for future studies, particularly if the analyte list for chemicals of emerging interest is expanded.

8.0 SUMMARY AND CONCLUSIONS

8.1 Introduction

This report documents the approach, results, and interpretation of a collaborative research study conducted by EPA's National Risk Management Research Laboratory, USDA, NCDA&CS, Battelle, and other organizations to evaluate the land application of Class B biosolids The overall goal of this research was to investigate air, volatile emissions, and soil sampling methods and analytical techniques. To accomplish this goal, samples were collected using a variety of sampling methods and equipment and analyzed for a broad matrix of chemical, physical, and microbial species. It is anticipated this study along with other research will eventually lead to the development of a standardized protocol that can be used in future studies on the application of biosolids to land.

This research was conducted under EPA Quality Assurance Project Plan (QAPP) No.163-Q10-2, and it represents the first known comprehensive study evaluating this variety of sampling methods and analytical techniques simultaneously in the field before, during, and after the land application of biosolids. The study commenced in August 2004 at the NCDA&CS Piedmont Research Station in Salisbury, NC. Biosolids application was conducted in September 2004, and field monitoring continued until January 2, 2005.

8.2 Study Description

Land application occurred on a fescue field with no previous exposure to biosolids. Other than modifications to facilitate sampling, application practices and equipment were typical of those used during normal agronomic biosolids application. Biosolids were land applied at a target rate of 10 wet tons/acre.

Measurements were made of air emissions (volatile odorants and microorganisms) and their short-range transport; airborne particulates; and soil microbial and chemical concentrations at and around the test site before, during, and after biosolids application. To achieve the overarching goal of the project, the research was implemented via three tasks, each with its own discrete goals and sets of hypotheses:

Task 1. Bioaerosol and Particulate Matter Sampling. Select bacteria, fungi, viruses, bacterial endotoxins, and particulates were sampled in the aerosol emissions from biosolids prior to, during, and after biosolids application. The primary objectives for Task 1 were to: 1) characterize the types and measure the concentrations of the suite of viable bioaerosol components (seven bacteria, enteroviruses, and male-specific coliphage) and particulates; 2) determine if these bioaerosol components were emitted and transported to several points downwind of the biosolids application area under the circumstances investigated; and 3) evaluate the collection performance of the six-stage impactors, biosamplers, and GRIMM sampler in this field application study.

Task 2. Volatile Organic Carbon and Odorant Monitoring and Analysis. The presence and concentration of a selected group of inorganic and organic compounds and odorants were measured in emissions generated upwind, within, and downwind of the application area of the biosolids land application test site. The objectives of this task were to: 1) quantify the concentrations of specific compounds identified in the emissions including VOCs and odorants; and 2) determine the transport (if any) of these chemicals downwind of the biosolids application area at this site.

Task 3. Land Sampling. The soil sampling component of this research involved a longer sampling period than did other tasks and focused on measuring the concentrations of microbes and

chemicals before and for 4 months after biosolids application. The specific objectives for Task 3 were to: 1) characterize the quantity and distribution of biosolids applied, 2) characterize the microbial community quantity and structure in the soil, 3) measure the fecal coliform concentrations in the soil, 4) measure the concentration of alkylphenols and alkylphenol ethoxylates (APEs) in the soil, and 5) screen soil samples for terrestrial ecotoxicity.

In addition to the work described here, two additional studies were carried out during the biosolids land application and one study was completed in the spring of 2005 following biosolids application at the site. These investigations were not part of the project-specific quality assurance plan and, therefore, are not reported in the body of this report. The work, however, is related to this study, and the results are provided in the report appendices as follows: Appendix A. *Determination of Total Bacterial Bioburden from Impinger Samples Collected During the NC Biosolids Land Application Study – Dr. Mark Hernandez, University of Colorado*; Appendix B. *Parallel Sampling Approaches and Analysis of Impinger Samples Collected During the NC Biosolids Land Application Study – Dr. Ian Pepper, University of Arizona;* and Appendix C. *Endotoxin Sampling During a Post-Spring Cutting Event at the NC Biosolids Land Application Study Site – Dr. Edwin Barth, EPA/NRMRL*.

8.3 Study Site

The test site, shown previously in Figure 2.2, consisted of a 100-m diameter circle application area (approximately 2 acres) located within the selected fescue field. It was designed to accommodate an array of upwind and downwind bioiaerosol sampling units that could be moved around a circular center point if needed due to changes in wind direction during application. A total of nine stationary sampling locations were positioned on each of three parallel sampling lines (three per line), one upwind and two downwind. In addition to the stationary sampling locations, one mobile sampler (MOB) consisting of an ATV equipped with a similar sampling equipment array was deployed to follow within 15 m of the application plume behind the hopper.

8.4 Applied Biosolids

The Class B biosolids applied during the study consisted of anaerobically digested and mechanically dewatered (centrifugation combined with polymer addition) municipal wastewater treatment plant sludge. At the request of the researchers, this material was pretreated with only enough lime to adjust the pH and suppress microbial growth to meet facility compliance for release (material met Class B compliance at time of release from facility). Heavier doses of lime are more consistent with the normal operation of the facility. Therefore, this material was atypical of biosolids that would normally be released from this facility. As a result of this modified sludge treatment regimen, researchers hoped that viable microbial populations would be available in the aerosol phase for collection and analysis once the study was implemented. This specially prepared/treated sewage sludge was also expected to elicit odors and generate particulates via surface drying, flaking, and wind erosion during land application. The parameters measured in the biosolids were consistent with those used throughout the investigation for comparison purposes.

8.5 Field Results

In addition to characterizing the applied biosolids, researchers simultaneously conducted bioaerosol sampling, volatile organic and inorganic compounds collection and analysis, odorant monitoring and sampling, and land sampling activities within and near the application test area prior to, during, and following land application. A comprehensive description of all sample collection procedures and analytical methodologies can be found in Section 2.0.

The measurements conducted and the analytical data generated on this project are discussed in detail in Section 4.0 for biosolids characterization, Section 5.0 for bioaerosol and particulate sampling, Section 6.0 for volatile organic and inorganic emissions and odorant sampling, and Section 7.0 for land sampling. Key results and conclusions are summarized below in Sections 8.5.1, 8.5.2, 8.5.3, and 8.5.4, respectively.

8.5.1 Biosolids Characterization. The biosolids used in this study had a solids content of 28% and contained 2.3×10^9 CFU fecal coliforms/g dry weight (gdw) total solids and 6.33 most probable number (MPN) *Salmonella* spp./gdw total solids. Microbial measurements included total heterotrophic bacteria (THB) at 1.6×10^{11} CFU/gdw total solids, *Escherichia coli* at 4.35×10^7 MPN/gdw total solids, total coliforms at 1.4×10^9 CFU/gdw total solids, and *Enterococcus* spp. at 8.2×10^5 CFU/gdw total solids. Samples were assayed for *Staphylococcus aureus*, but none were detected. In addition, a number of organic SVOCs and VOCs were measured in the stockpile prior to application.

8.5.2 Bioaerosol and Particulate Sampling. In the aerosol phase of this study, THB, which include the saprophytic aerobes and facultative anaerobes that are naturally present in soil, on plant surfaces, in air, and in water, were detected in all biosamplers and on agar plates in all six-stage impactors for both the control trial and biosolids application test. Their presence in air samples upwind and downwind demonstrated that the air samplers were operating sufficiently well to collect viable microbes. Fungi were also assayed and detected in all six-stage impactors.

THB and the fungi data were evaluated to determine if there were significant differences in the THB and total fungi counts between: 1) upwind and downwind locations and 2) the MOB and other locations. In addition, apparent differences in THB and fungi data between the control trial and the biosolids application test were compared. For Comparison 1, statistically significant differences in THB concentrations were observed between the upwind sampling transect and the farthest downwind sampling transect during the application of biosolids to the land. Similar behavior was observed for fungi data. However, no statistically significant differences were observed during the control trial. For Comparison 2, multiple statistically significant differences for THB were observed between the MOB and the stationary sampling locations. Once again, fungi results were similar to the THB observations. These observations were noted in both the control trial and application test. No apparent differences in counts were observed between the control trial and the biosolids application test for THB and fungi data.

Detection of pathogenic and indicator microorganisms was also anticipated due to the bacterial counts observed in the bulk biosolids. However, fecal coliforms, *E.coli*, *Salmonella* spp., *S. aureus*, *C. perfringens*, *Enterococcus* spp., and coliphage were not detected in any of the bioaerosol samples collected at the stationary sampling locations on the site either in the control trial or during biosolids application. These organisms also were not detected in any of the samples collected by the MOB.

Enteric virus analyses were conducted using the plaque forming unit (PFU) and MPN procedures initially on samples collected from mid-line stationary samplers and MOB for the control trial and application test. No positive results were found for enteric virus in the PFU and MPN analyses conducted on these initial samples. Therefore, no further viral analyses were conducted on any additional samples.

Total mass concentrations ($\mu g/m^3$) of particulates ≤ 5.0 μm were measured using the GRIMM particle analyzer. Particulate mass increased by approximately 90 $\mu g/m^3$ during the onset of field activity during the control trial. No statistical differences in mass were noted either between samples collected during the control trial and the biosolids application sampling period or between samples taken immediately before and during biosolids application.

8.5.3 Volatile Organic and Inorganic Emissions and Odorant Sampling. Headspace analyses indicated that detectable levels of acetone, 2-butanone, methylene chloride, toluene, dimethyl sulfide, and dimethyl disulfide were associated with the biosolids removed from the stockpile and applied to the field. Among the volatile compounds, the estimated emission factor was highest for dimethyl sulfide (range of 230 to 660 ng/g wet solids) for all three sampling periods (0 hr, 24 hr, and 48 hr). The emissions factor for all of the compounds detected decreased for each of the 2 days following application, except for dimethyl disulfide, which remained relatively constant or showed a slight increase over time. While methylene chloride was suspected as a laboratory contaminant, the other detected compounds are likely organic byproducts of the anaerobic digestion of municipal biosolids typically found in very low concentrations emitting from biosolids as volatile gases.

Exposure of solid-phase microextraction (SPME) fibers to the headspace of biosolids for 1 hr also resulted in detectable concentrations of dimethyl sulfide (1.8 to 8.0 ppmv) and dimethyl disulfide (0.75 to 2.0 ppmv). Trace levels (0.25 ppmv) of carbon disulfide also appeared in the SPMEs only in the control trial. No significant trend was observed in the SPME headspace results for the time period samples (i.e., 1 hr, 24 hr, and 48 hr after biosolids application).

Flux chamber air emissions (collected within Summa canisters and analyzed by gas chromatography/mass spectrophotometry) produced detectable concentrations for acetone, trimethylamine, dimethyl sulfide, and dimethyl disulfide. Estimated flux rates for these compounds were greater than 1.0 $\mu g/m^2/hr$ for several of the post-application sampling times (0, 3, 4 hr, and 20 hr). The rate of flux chamber emissions declined with time after biosolids placement. Dimethyl sulfide and dimethyl disulfide emissions persisted into the 20th hour after biosolids application when sampling was terminated. Flux chamber information should be considered carefully as temperatures within the flux chambers were often higher than ambient temperatures. Since higher temperatures may enhance volatility and chemical reactions, this emission information may not be representative of biosolids land application.

SPME results obtained from flux chamber samples have been treated as semi-quantitative since significant holding time delays occurred. Comparison of results between the SPME samples collected in Tedlar® bags and the Summa canister method analyzed by GC/MS indicated that dimethyl sulfide, dimethyl disulfide, and trimethylamine were found consistently with both methods. However, concentrations determined with the canister method were an order of magnitude higher than those produced with the SPMEs, indicating potential losses over time or other interferences resulting in inadequate sorption onto the SPME fiber.

Odor was monitored in the field using Nasal Rangers®. The dilution at which the odor was barely detected, i.e., the detection threshold (DT), was determined using ASTM E544-99 (ASTM, 2004). Odor units were expressed as the number of dilutions-to-threshold (d/T) before odor was barely detected. Odorous materials have smaller d/T values than do less odorous materials. For the on-site monitoring conducted using Nasal Rangers®, DTs during biosolids application were 15 to 30 d/T approximately 25 m downwind of the application site and 2 to 7 d/T approximately 25 m upwind of the application area. At distances greater than 75 m, odors were undetected at both upwind and downwind locations. Approximately 22 hr after biosolids application, odor levels in the application area were comparable to the levels at 25 m upwind and downwind during application, and odor was undetectable above background levels elsewhere in the project area. After 48 hr, odors were barely detectable downwind of the site, and after 196 hr, no odors were detected above background in any location on the site.

Additional samples were collected from flux chamber exhaust in 12-L Tedlar® bags for shipment to a laboratory and off-site olfactometry analysis to confirm odor presence and determine odor concentrations using ASTM E679-91 (ASTM, 1991). Substantially increasing odors were detected by the off-site panel on samples collected during biosolids application up to 22 hr after application. It is

believed the increasing odors noted into the afternoon and throughout the day of application were an artifact of the flux chamber units. Solar heating elevated air temperatures inside the chambers, thereby increasing volatility and anaerobic degradation of organic sulfur compounds contained in the applied biosolids. Therefore, while flux chambers are useful for estimating odor generation during and for perhaps up to an hour after biosolids application, they are not considered practical, effective, and accurate for determining odor levels over longer sampling periods stretching into multiple hours and days.

In-field measurements of ammonia and hydrogen sulfide were conducted using Draeger tubes and the Jerome® analyzer, respectively. Sampling locations were identified during the Nasal Ranger® monitoring activities. Ammonia was not detected in above-ground air samples during the control trial within the application area. Immediately after the application test, ammonia was detected at concentrations of 0.10 to 0.90 ppmv for near-ground samples within the application area and from a flux chamber exhaust sample at 15 ppmv. Within the application area, hydrogen sulfide was detected at levels near the recognition threshold, 0.002 to 0.050 ppmv during the control trial and 0.007 to 0.021 ppmv directly behind the moving biosolids application equipment during biosolids application. The exhaust from the biosolids applicator machinery may have been responsible for some of the hydrogen sulfide gas measured. Immediately after the biosolids application test, hydrogen sulfide was detected within the application area at slightly lower concentrations than those detected during the control trial (within the range of 0.001 to 0.007 ppmv for near-ground air samples) and from a flux chamber exhaust sample at 0.160 ppmv. The highest measurements for each of the gases never approached any health criterion or guidance level, and both gases were below detectable limits (0.001 ppmv) 400 m downwind of the application area during the application trial. Due to their high vapor pressures and the existing field conditions, the concentration of ammonia and hydrogen sulfide decreased by the second day after application and were below detectable limits within 4 days.

The OP-FTIR also confirmed the presence of ammonia, and the data were used to calculate a flux emission rate across the site after the addition of biosolids to the test area. Immediately after biosolids addition and upwind of the application area, the calculated ammonia flux rate was 0.006 g/s while downwind it was 0.063 g/s. In order to investigate the rate of emissions decay, measurements continued for several hours after biosolids application. The upwind VRPM configuration measured negligible ammonia concentrations during the post-application period. However, the downwind VRPM configuration detected ammonia plumes for several hours after the application ended. The calculated emission flux from the reconstructed ammonia plume was 0.036 g/s at 2 hr after application, decreasing further to 0.009 g/s approximately 3 hr after application.

8.5.4 Land Sampling. The soil sampling portion of this study focused on the concentrations of microbes and chemicals found in the soil matrix prior to and for 4 months following the applications of biosolids. The quantity and distribution of applied biosolids were characterized by measuring dry mass and ash mass in three replicate plots. Statistical analysis determined that biosolids were evenly distributed in two plots at 10.8 ± 4.4 g ash/900 cm^2. In the other plot, biosolids distribution was uneven at 20.0 ± 5.6 g ash/900 cm^2. These data and further statistical analysis are consistent with qualitative observations made in the field (see Section 7.0 for photographs showing the distribution of biosolids at the test site). Measured wet and dry masses were used to calculate the amount of biosolids applied, 7.3 to 9.5 wet tons/acre, which is equivalent to 1.7 to 2.2 dry tons/acre. This measured application rate was slightly lower but in the range of the planned agronomic application rate of 10 wet tons/acre.

The quantity and diversity of the microbial community were characterized at three depths. Total biomass was measured based on the quantity of lipid phosphate in a sample, while community structure, a measure of microbial diversity, was characterized by the relative abundance of individual PLFAs. Microbes produce different PLFAs depending on the types of organisms present and environmental conditions. Biosolids application may alter the microbial community of the soil by adding nutrients,

organic matter, chemicals, and microbes. PLFA-based community structure assessments displayed small changes correlating to changes in soil conditions, such as the rainfall immediately prior to biosolids application. More substantial changes were observed following biosolids application. However, by Day 28, community structure was similar to its pre-application state.

The PLFA-based biomass measurements documented differences as a function of soil depth with the majority of the microbial mass occurring within the top 5 cm of the soil matrix. Biosolids application increased the total biomass by 60 nmol/g dry weight on average. Sample variability, often around 30%, limited the statistical conclusions that could be drawn. Post-application levels were stable from Day 28 through the end of the sampling period at Day 98. Statistically significant differences were observed between plots.

Assessments of pathogenic organisms relied on measurement of fecal coliforms as an indicator organism. A limited number of samples were also analyzed for enteric viruses, *Salmonella* spp., and viable Helminth ova (VHO). Semi-quantitative fecal coliform data were reported by the analytical laboratory, complicating statistical evaluations. Fecal coliform density displayed a statistically significant increase of more than 100-fold between pre-application and post-application samples. The post-application density generally persisted through Day 98 following application. About 20% of the samples contained detectable concentrations of enteric viruses, *Salmonella* spp., and VHO prior to, during, and after biosolids application.

APEs and their metabolites OP and NP were of interest as potential endocrine disrupting chemicals (EDCs). APEs were not detected in the soil at any time during the sampling period. OP and NP were observed frequently following application in shallow samples but not in deeper samples. Downward migration of these compounds may have been affected by the physical/chemical structure of the biosolids, the affinity of these compounds for the biosolids matrix, and the high clay content of the soil in this study. Variability in OP and NP concentrations was too high to identify temporal changes.

Ecotoxicity was screened using earthworm mortality and 5-day seed germination and root elongation assays with lettuce and oats. Results did not exhibit a consistent pattern. Earthworm mortality and seed germination exhibited no changes following biosolids application. Since both assays indicated maximal response prior to application, no added benefit of biosolids could be demonstrated. Neither of the above assays produced a negative response to biosolids. Lettuce root length, however, demonstrated enhanced growth following biosolids application, while oat root length showed reduced growth.

8.6 Lessons Learned

Implementing this large-scale and fairly complex strategy for sampling during an active biosolids land application event had inherent challenges, and difficulties were encountered during the field trial that may have influenced the final results. The study relied on meteorological data collected in the northeast quadrant of the test area. The wind direction varied substantially between the control trial and application test, while the velocity varied only slightly. Subtle changes may have been observed by operators across the application area. In retrospect, real-time wind direction and wind speed recordings within individual transects or even stations would have been informative and may have helped in the interpretation of the variability observed between individual stations on the same and/or differing transect lines.

Even though two-way radios and headsets were used by operators at each station, communication among personnel assigned to each sampling station was still relatively ineffective, primarily due to noise and obstruction of view from the applicator. This situation may have resulted in slight discrepancies in sampling times on sample lines and did not permit the samplers to be repositioned into the prevailing wind direction during wind shifts as originally planned.

Particulate matter was only recorded in the center of the application area and was presumed to travel some distance downwind. Future studies should consider the use of real-time particulate analyzers in the upwind and downwind sampling locations. The dataset produced from this bioaerosol sampling effort was highly variable.

The study was designed to accommodate a number of different objectives for other specific tasks in addition to the aerosol sampling component. For example, it was of interest, particularly for Task 3, to conduct this work at a site where biosolids had never been land applied. However, based on the variability observed in this trial, the bioaerosol sampling component would have benefited from replicated application tests and subsequent focused sampling events to increase the number of observations and reduce uncertainties.

In retrospect, the bioaerosol sampling design may have benefited from the use of air dispersion modeling once the application site was identified and predominant weather conditions could be estimated. When sampling for studies that span multiple days, care should be taken to collect the samples during the same approximate time intervals each day. This practice may help decrease the impacts that may be caused by variable environmental conditions, such as temperature, wind direction, humidity, etc.

The nature of the biosolids used may have had a significant impact on the data generated in this study. The biosolids were sticky and cohesive, possibly due to polymer addition during sludge dewatering or other sludge processing operations. It is believed the viscid nature of the biosolids substantially reduced their friability and perhaps limited their ability to be dispersed as fine particles into the air. Consequently, the biosolids (when slung from the application vehicle and applied to the test site) tended to settle onto the ground in small agglomerated clumps rather than as discrete particles. This characteristic visually impacted the distribution of the biosolids on the soil surface and was not anticipated in the soil/biosolids sample collection plan. Further, the cohesive nature of the biosolids may have decreased the number of particles aerosolized and, consequently, the capture and detection of aerosolized microorganisms. For future studies in which the primary objective is to maximize dispersion of biosolids and associated chemicals and microorganisms, it is recommended that preliminary screening evaluate biosolids friability and likely distribution into the air. Application of liquid biosolids may also yield a more uniform distribution of droplets to the soil and into the air. In this study, application of a more agglomerating biosolids product appears to have restricted the applied material to the immediate area and limited the spread of airborne particles to downwind receptors.

8.7 Recommendations

Additional work will be needed in order to develop a detailed protocol for future biosolids land application studies. At the completion of this study, the following recommendations, coupled with suggestions for improvement where appropriate, can be offered for similar activities:

8.7.1 Bioaerosol Sampling. Careful consideration should be given to the analyte list when designing a bioaerosol sampling protocol. The customarily-used sampling devices have significant limitations in enabling the conduct of a comprehensive bioaerosol sampling program, particularly for capturing stress-sensitive pathogenic bacteria. Until such time as more robust equipment is designed and developed specifically for outdoor applications, the types and species of microorganisms and/or indicators selected for assay should be those that are relevant to project objectives but also relatively easy to culture.

Meteorological data such as wind velocity and patterns should be acquired for a substantial period prior to the study and used to develop predictive models, which in turn could be used to develop and optimize bioaerosol sampling array designs. Ultimately, development of a sampling array design that is independent of wind direction (i.e., a design that does not need to consider a shift in sampling locations to

acquire representative samples) is a desirable goal. Also, incorporation of elevated samplers in the sampling array design to accommodate capture of vertically dispersed bioaerosols would enhance the overall comprehensiveness of the design.

In addition, when sampling aerosols at time intervals for studies that span multiple days, care should be taken to collect the samples at the same approximate time period each day. This practice may help decrease the impacts that may be caused by variable environmental conditions, such as temperature, wind direction, humidity, etc.

Endotoxin sampling may be useful to indicate the bioaerosol emissions associated with biosolids and should be evaluated further. Even though analytical, logistical, and quality control problems made it impossible to interpret data from this study, endotoxins are generally easy to sample and may be present in sufficiently high numbers to permit the conduct of a statistical analysis.

8.7.2 Particulate Sampling. Particulate sampling should be conducted both upwind and downwind of biosolids application areas and in close association with bioaerosol samplers so that bioaerosol and particulate data can be correlated. The design implemented in this study, which confined particulate sampling to the center of the application area, did not allow for this correlation of data. The GRIMM particle analyzer was utilized in this study to develop mean mass information on airborne particulates for different phases of the project. For future studies, this particulate monitoring device could also be used to take advantage of its real-time data acquisition capabilities to facilitate in-field decisions such as placement or movement of aerosol sampling equipment.

8.7.3 Volatile Organic Compound and Ammonia Sampling. Collecting samples using VRPM via an OP-FTIR spectrophotometer is expensive and complex, but this technique results in a three-dimensional map of plumes emanating from biosolids land application. From these measurements, chemical-specific flux estimates can be determined. In this study, ammonia plumes were detected during and following biosolids application, but not during baseline sampling. No interpretive data were developed regarding VOCs during the study. Given these results, while use of this technique cannot be conclusively recommended for detecting and quantifying VOCs, it‘s use can be recommended for producing real-time estimates of ammonia plume generation and dissipation.

Flux chambers used in combination with Summa canisters can also be used to estimate VOC emissions; however, internal chamber temperatures can become elevated influencing chemical volatilization and microbial activity, and possibly biasing emission results. It is recommended that future studies employ the use of Summa canisters without flux chambers and that they be positioned in upwind and downwind locations, as well as in the application area. Predictive models can also be used to identify appropriate Summa canister stations. SPME-based analytical methods may also be useful, but care should be exercised to stay within sample holding times.

Ammonia sampling using Draeger tubes and hydrogen sulfide measurements using the Jerome analyzer provided inexpensive and reliable measurements of these two odiferous compounds at specific locations downwind of the biosolids application site. These measurements provided useful information regarding the geographic distribution of odiferous chemicals in the area surrounding the biosolids land application site.

8.7.4 Odor Sampling. Field olfactometry using on-site Nasal Rangers® is an acceptable method to establish real-time geographic distribution of odor and worked well for this study. The protocol would likely benefit from sampling for a longer duration to account for temporal changes due to local area impacts such as changing temperature, humidity, or wind direction. As discussed earlier, flux chamber samples may have been influenced by higher internal temperatures. Nasal Rangers® are more flexible,

effective, and accurate for determining on-site near- and far-field odor levels over extended sampling periods.

8.7.5 Land Sampling. A successfully implemented field protocol for determining the effects of biosolids on land is dependent on a carefully designed statistical approach due to the inherent heterogeneity of the microbial population in the soil. The use of replicate sample plots and replicate sampling within plots is strongly recommended to facilitate separation of plot effects from time or other variables for statistical analysis.

Since biosolids may not be applied to the land uniformly, it is necessary to determine the spatial distribution of the biosolids material as applied. The method of pinning geotextile fabric to the ground to collect biosolids mass and distribution samples for this study worked quite well and is recommended for additional studies.

PLFA measurements reliably track changes in the soil microbial community that result from biosolids land application. Because PLFA results vary significantly by depth, it is important that sufficient numbers and types of samples be collected in order to facilitate the statistical analyses needed to identify differences that are important to the study. Furthermore, the results from this study would suggest using an approach that focuses sample replication in the shallow soil horizon as minimal downward analyte migration was observed. However, this observation may have been impacted by the high level of fine-grain particles (clay) present in the soil at this site.

The reliance on fecal coliforms as an indicator organism was useful as detection frequency was high enough to facilitate interpretative analysis of the data. However, due to data quality problems, uncertainties in the conclusions that could be drawn from the fecal coliform data were substantial. Follow-on studies are recommended. More sample replicates within a plot, more replicate plots, and more precise data from the analytical laboratory may reduce these uncertainties. In addition, extending the sampling period following biosolids application may be useful.

The results of the APE testing indicated that the analytical methods used were appropriate for biosolids and biosolids/soil mixtures. However, since APEs persisted in the soil matrix for the duration of the study, a longer sampling period may be needed for future projects. Due to the variability in data observed at this site, future APE sampling designs may benefit from a different approach to sampling that considers such options as larger samples with homogenization, sample or extract composites, or normalization with a biosolids marker. Finally, expanding the list of EDC analytes to include a broader spectrum of chemicals is recommended.

Toxicity screening produced information characterizing the response of selected organisms to biosolids application and is recommended for future studies. In addition to the assays used in this study, chronic earthworm tests with non-lethal endpoints, such as biomass accumulation, and longer-term plant studies would be useful in gaining a fuller understanding of the effects of biosolids. A longer sampling period may also be useful.

Soil characterization was relatively inexpensive compared to many of the other analyses conducted and provided useful supporting data for this study. This characterization, particularly for sites such as this one with high soil clay content, should be focused on the shallower depth horizon due to microbial population dynamics. Similarly, weather data collection was relatively inexpensive and provided useful information.

9.0 REFERENCES

Acheson, C.M., Q. Zhou, Y. Shan, G.D. Sayles, and M.J. Kupferle. 2004. "Comparing the Solid Phase and Saline Extract Microtox(R) Assays for Two Polycyclic Aromatic Hydrocarbon-Contaminated Soils," *Environmental Toxicology and Chemistry*. 23, 245-251.

Anderson, T.D. and J.D. MacRae. 2006. "Polybrominated Diphenyl Ethers in Fish and Wastewater Samples from an Area of the Penobscot River in Central Maine," *Chemosphere*. 62, Issue 7, 1153-1160.

ASTM Standard E679-91. 1991. "Standard Practice for Determination of Odor and Taste Thresholds by a Forced-Choice Ascending Concentration Series Method of Limits," ASTM International, West Conshohocken, PA, www.astm.org.

ASTM Standard E544-99. 2004. "Standard Practices for Referencing Suprathreshold Odor Intensity," ASTM International, West Conshohocken, PA, www.astm.org.

APHA. 1992. Method 9215 C. "Spread Plate Method". *Standard Methods for the Examination of Water and Wastewater*, 17th Edition, Washington, DC.

Baertsch, C., T. Paez-Rubio, E. Viau, and J. Peccia. 2007. "Source Tracking of Aerosols released from Land-Applied Class B Biosolids During High-Wind Events," *Applied and Environmental Microbiology*. 739 (14), 4522-4531.

Banks, M.K., A.P. Schwab, N. Cofield, J.E. Alleman, M. Switzenbaum, J. Shalabi, and P. Williams. 2006. "Biosolids Amended Soils: Part I. Effect of Biosolids Application on Soil Quality and Ecotoxicity," *Water Environ. Res*. 78, 2217-2230.

Beebe, K.R., R.J. Pell, and M. Seashultz. 1998. *Chemometrics: A Practical Guide*, John Wiley and Sons, New York, NY.

Brooks, J, B. Tanner, K. Josephon, C. Gerba, C. Haas, and I. Pepper. 2005. "A National Study on the Residential Impact of Biological Aerosols from the Land Application of Biosolids," *Journal of Applied Microbiolg.* 98(2), 397-405.

Brooks, J., B. Tanner, K. Josephson, C. Gerba, and I. Pepper. 2004. "Bioaerosols from the Land Application of Biosolids in the Desert Southwest USA," *Water Science and Technology*. 50(1), 7-12.

Barth, E. 2007. "Lessons Learned Regarding the Use of an Impinger for Collecting Airborne Bacteria during a Biosolids Application Field Study." *Proceedings of the Biosolids Exposure Measurement Workshop*, Office of Research and Development, EPA, Cincinnati, OH.

Battelle. 2004. *Final Quality Assurance Project Plan (QAPP) for Multi-Media Sampling at Biosolids Land Application Sites* #390-QC-1. September.

Chang, L.W., J.R. Meier, and M.K. Smith. 1997. "Application of Plant and Earthworm Bioassays to Evaluate Remediation of a Lead-contaminated Soil," *Arch. Environ. Contamin. Tox*. 32, 166-171.

Dettenmaier, E. and W.J. Doucette. 2007. "Mineralization and Plant Uptake of ^{14}C-labelled Nonylphenol, Nonylphenol Tetraethoxylate and Nonylphenol Nonylethoxylate in Biosolids/Soil Systems Planted with Crested Wheatgrass," *Environmental Toxicology and Chemistry*. 26, 193-200.

Dorn, P.B., T.E. Vipond, J.P. Salanitro, and H.L. Wisniewski. 1998. "Assessment of the Acute Toxicity of Crude Oils in Soils Using Earthworms, Microtox®, and Plants," *Chemosphere.* 37, 845-860.

Environment Canada. 1994. *A Review of Whole Organism Bioassays for Assessing the Quality of Soil, Freshwater Sediment, and Freshwater in Canada (Scientific Series No. 198)*, Ottawa, ON.

Fabian, M., S. Miller, T. Reponen, and M. Hernandez. 2005. "Ambient Bioaerosol Indices for Indoor Air Quality Assessments of Flood Reclamation," *Aerosol Science*, 36, pp. 763-783.

Gabriel, S., S. Vilalai, S., C. Peot, and M. Ramirez. 2006. "Statistical Modeling to Forecast Odor Levels of Biosolids Applied to Reuse Sites," *Journal of Environmental Engineering*, 132(5), 479-488.

Hamel, K., L. Walters, C. Sulerud, and M. McGinley. 2004. "Land Application Odor Control Case Study," Water Environmental Federation Residuals and Biosolids Management Conference, Salt Lake City, Utah.

Heidelberg, J., M. Shahamat, M. Levin, I. Rahman, G. Stelma, C. Grim, and R. Colwell. 1997. "Effect of Aerosolization on Culturability and Viability of Gram-Negative Bacteria," *Applied and Environmental Microbiology*, 63(9), pp. 3585-3588.

Hesselsoe, M., D. Jensen, K. Skals, T. Olesen, P. Moldrup, P. Roslev, G.K. Mortensen, and K. Henriksen. 2001. "Degradation of 4-Nonylphenol in Homogeneous and Nonhomogeneous Mixtures of Soil and Sewage Sludge," *Environ. Sci. Technol*. 35, 3695-3700.

Hirst, J.M. 1995. "Bioaerosols: Introduction, Retrospect and Prospect," C.S. Cox and C.M. Wathes, eds., *Bioaerosols Handbook*. CRC Press, Inc., Lewis Publishers, Boca Raton, FL, pp. 5-25.

Hseu, Z.Y. 2006. "Response of Microbial Activities in Two Contrasting Soils to 4-nonylphenol Treated with Biosolids," *Chemosphere.* 64, 1769-1776.

Hund, K. and W. Traunsurger. 1994. "Ecotox-evaluation Strategy for Soil Bioremediation Exemplified for a PAH-contaminated Site," *Chemosphere.* 29, 371-390.

Kaur, A., A. Chaudhary, A. Kaur, R. Choudhary, and R. Kaushik. 2005. "Phospholipid Fatty Acid – A Bioindicator of Environment Monitoring and Assessment in Soil Ecosystem," *Current Science.* 89, 1103-1112.

Kinney, C.A., E.T. Furlong, D.W. Kolpin, M.R. Burkhardt, S.D. Zaugg, S.L. Werner, J.P. Bossio, and M.J. Benotti. 2008. "Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended with Biosolid or Swine Manure," *Environ. Sci. Technol*. 42(6), 1863-1870.

Krach, K., B. Burns, B. Li, A. Shuler, C. Colee, and Y. Xie. 2008. "Odor Control for Land Application of Lime Stabilized Biosolids," *Water Air & Soil Pollution: Focus*. 8(3-4), 368-378.

Low, S., T. Paez-Rubio, C. Baertsch, M. Kucharski, and J. Peccia. 2007. "Off-site Exposure to Respirable Aerosols Produced During the Disk-Incorporation of Class B Biosolids," *Journal of Environmental Engineering*. 133(10), 987-994.

Macher, J.M. 1989. Positive-hole Correction of Multiple-jet Impactors for Collecting Viable Microorganisms. *Amer. Inc. Hyg. Assoc. J.* 50, 561-568.

Marcomini, A., P.D. Capel, Th. Lichtenseiger, P.H. Brunner, and W. Giger. 1989. "Behaviour of Aromatic Surfactants and PCBs in Sludge-treated Soil and Landfills," *Journal of Environmental Quality*. 18, 523-528.

Meier, J.R., L.W. Chang, S. Jacobs, J. Torisella, M.C. Meckes, and M.K. Smith. 1997. "Use of Plant and Earthworm Bioassays to Evaluate Remediation of Soil from a Site Contaminated with Polychlorinated Biphenyls," *Environ. Tox. Chem.* 16, 928-938.

Mortensen, G.K. and L.K. Kure. 2003. "Degradation of Nonylphenol in Spiked Soils and in Soils Treated with Organic Waste Products," *Environ. Tox. Chem.* 22, 718-721.

Mocé-Llivina, L., M. Muniesa, M. Pimenta-Vale, F. Lucena, and J. Jofre. 2003. "Survival of Bacterial Indicator Species and Bacteriophages after Thermal Treatment of Sludge and Sewage." *Appl. Environ. Microbiol.* 69, 1452-1456.

National Research Council (NRC). 2002. *Biosolids Applied to Land — Advancing Standards and Practices*, The National Academies Press, Washington, DC.

NIOSH, 1998. Biosolids Land Application Process - LeSourdsville, OH. HETA 98-0118-2748, Cincinnati, OH

Paez-Rubio, T., A. Ramarui, J. Sommer, H. Xin, and J. Peccia. 2007. "Emission Rates and Characterization of Aerosols Produced During the Spreading of Class B Biosolids," *Environmental Science and Technology*. 41, 3537-3544.

Peacock, A.D., M.D. Mullen, D.B. Ringelberg, D.D. Tyler, D.B. Hedrick, P.M. Gale, and D.C. White. 2001. "Soil Microbial Community Responses to Dairy Manure or Ammonium Nitrate Applications," *Soil Biology and Biochemistry*. 33, 1011-1019.

Pillai, S., K. Widmer, S. Dowd, and S. Ricke. 1996. "Occurrence of Airborne Bacteria and Pathogen Indicators during Land Application of Sewage Sludge," *Applied Environmental Microbiology*. 62(1), 296-299.

Rusin, P., S. Maxwell, J. Brooks, C. Gerba, and I. Pepper. 2003. "Evidence for the Absence of *Staphylococcus aureus* in Land Applied Biosolids," *Environmental Science and Technology*. 37, 4027-4030.

Salintro, J.P., P.B. Dorn, M.H. Huesemann, K.O. Moore, I.A. Rhodes, L.M. Rice Jackson, T.E. Vipond, M.M. Western, and H.L. Wisniewski. 1997. "Crude Oil Hydrocarbon Bioremediation and Soil Ecotoxicity Assessment," *Environ. Sci. Technol*. 31, 1796.

Scott, T.M., J.B. Rose, T.M. Jenkins, S.R. Farrah, and J. Lukasik. 2002. "Microbial Source Tracking: Current Methodology and Future Directions." *Appl. Environ. Microbiol*. 68: 5796-5803.

Statistical Analysis Software. 2003. SAS Version 9.1, Cary, NC.

Simini, M., R.S. Wentsel, R.T. Checkai, C.T. Phillips, N.A. Chester, M.A. Major, and J.C. Amos. 1995. "Evaluation of Soil Toxicity at Joliet Army Ammunition Plant," *Environ. Tox. Chem.* 14, 623-630.

Tanner, B., J. Brooks, C. Gerba, C. Haas, K. Josephson, and I. Pepper. 2008. "Estimated Occupational Risk from Bioaerosols Generated During land Application of Class B Biosolids," *Journal of Environmental Quality*. 37, 2311-2321.

Tanner, B., J. Brooks, C. Haas, C. Gerba, and I. Pepper. 2005. "Bioaerosol Emission Rate and Plume Characteristics during Land Application of Liquid Class B Biosolids." *Environ. Sci. Technol*. 396, 1584-1590.

Tanner, B., J. Brooks, C. Haas, C. Gerba, and I. Pepper. 2005b. "Bioaerosol Emission Rate and Plume Characteristics during Land Application of Liquid Class B Biosolids," *Environmental Science and Technology*. 396, 1584-1590.

U.S. Environmental Protection Agency (EPA). 1988. *Protocols for Short-Term Toxicity Screening of Hazardous Waste Sites*, EPA/600/3-88/029, Corvallis Environmental Research Laboratory: Corvallis, OR.

U.S. Environmental Protection Agency (EPA). 1989. *Ecological Assessment of Hazardous Waste Sites*, EPA/600/3-89/013, Corvallis Environmental Research Laboratory, Corvallis, OR.

U.S. Environmental Protection Agency (EPA). 1992. *Technical Support Document for Part 503 Pathogen and Vector Attraction Reduction Requirements in Sewage Sludge*, National Technical Information Service, NTIS No. PB89-136618, Springfield, VA.

U.S. Environmental Protection Agency (EPA). 1999. *Measurement of Volatile Organic Compounds in Biosolid Samples by Headspace Analysis Using Capillary Column Gas Chromatography/Mass Spectrometry (GC/MS)*.

U.S. Environmental Protection Agency (EPA). 2001. *Manual of Methods for Virology*, Chapter 15.

U.S. Environmental Protection Agency (EPA). 2003. *Environmental Regulations and Technology: Control of Pathogens and Vector Attraction in Sewage Sludge*, EPA/625/R-92/013.

Unc, A., J. Gardner, and S. Springthorpe. 2006. "Recovery of Soil *E. coli* after Addition of Sterile Organic Wastes," *Appl. Environ. Microbiol*. 72, 2287-2289.

Van Belle, G. and D.C. Martin. 1993. "Sample Size as a Function of Coefficient of Variation and Ratio of Means," *The American Statistician*. 47, 165-167.

Vestal, J.R. and D.C. White. 1989. "Lipid Analysis in Microbial Ecology," *Bioscience.* 39, 535-541.

Winfield, M.D. and E. Groisman. 2003. "Role of Non-host Environments in the Lifestyles of Salmonella and Escherichia coli," *Appl. Environ. Microbiol*. 69, 3687-3694.

APPENDIX A

Determination of Total Bacterial Bioburden from Impinger Samples Collected During the NC Biosolids Land Application Study – Dr. Mark Hernandez, University of Colorado

PROJECT REPORT

Molecular-based Identification of Bacterial Constituents from Aerosols Collected During the Land Application of Biosolids

Department of Civil, Environmental and Architectural Engineering, and Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder, University of Colorado, Boulder, Colorado, 80309

ABSTRACT

The ability of standard laboratory methods to detect microorganisms that could be potentially liberated to the environment during the land application of biosolids has not been well studied. Bacteria which may be aerosolized during the land application process, or during the subsequent weathering of biosolids, have only recently been investigated with modern genetic methods. The purpose of this study was to use the polymerase chain reaction (PCR) to amplify 16S rRNA genes for the detection of microbial indicators and/or pathogens commonly associated with biosolids. Air samples were obtained from liquid impingers immediately circumventing a well defined biosolids application area before, during and after spreading on a grass field. Samples were collected as close to the application vehicle as safety allowed. Liquid impinger samples were sent to the USDA microbiology laboratory for standard enrichments, and aliquots of those samples were processed to isolate and purify bacterial DNA, which was subsequently analyzed using broad-range rRNA PCR. Of 16 aerosol samples analyzed, 5 contained DNA that could be amplified by PCR. Genetic amplification detected an average of 35 different organisms in those 5 samples (range 28-51). In total, 439 rRNA clones were screened, and 157 phylotypes (DNA sequence relatedness groups) were identified. Using the most recent genetic library available from GenBank, the most abundant lineages/species were previously uncultured groups of bacteria that could not be classified by current systematic taxonomomy (18%), followed by a Beta Proteobacterium (6%), an uncultured L11 bacterium (4%), *Corynebacterium segmentosum* (3%), a *Ralstonia spp*. (3%), *Lactobacillus lactus* SL3 (3%), and a *Sphingomonas* sp. SKJH-30 (2%). Markedly less abundant species also were detected in some of the samples that may be considered as indicators or pathogens associated with sewage sludge, including two *Clostridium* species, several *Lactobacillus* species, and a CDC Group DF-3 isolate. Overall, the data demonstrated a low level of concordance with classical indicator organisms or pathogens associated with sewage sludge biosolids.

*Corresponding Investigator: Mark Hernandez,
Electronic mail: Mark.Hernandez@Colorado.Edu; Phone: 303 492 5991

INTRODUCTION

The aerosolization of bacteria from biosolids applications has long been a point of controversy and needs further investigation. Biosolids are repositories of both aerobic and anaerobic bacterial species, some of which are pathogenic to humans and other mammals. Whether standard culturing techniques can detect the full microbial array in biosolids as compared to newer molecular based techniques has not been fully investigated. Conventional laboratory techniques involve the use of bacterial plates with defined media that may also select for specific organisms. Less well understood is the symbiotic nature of mixed bacterial communities in biosolids with potential pathogens, and how land application and weathering may affect their aerosolization potential.

Knowledge of the complexities of the microbial communities found within biosolids is minimal. Traditionally, the identification and enumeration of microbial species from biosolids or their associated aerosols has depended entirely upon pure-culture techniques. However, the difficulty with which some types of microorganisms are cultured, particularly those with fastidious and/or anaerobic physiologies, means that the more easily grown species in a mixed microbial community likely are overrepresented by cultivation and plate counts. In many complex natural environments, for example, less than one percent of viable microbes present can be cultured under standard conditions (Pace, 1997).

Recently, culture-independent molecular methods of microbial identification and characterization have been developed and applied in the context of microbial ecology. Several of these techniques involve the use of ribosomal RNA (rRNA) gene sequences as tools for species identification by means of phylogenetic sequence analysis. rRNA genes can be amplified by polymerase chain reaction (PCR) directly from mixed-community DNA preparations, cloned, and individual clones sequenced. The occurrence of a particular rRNA gene sequence in a clone library indicates that the organism that encodes this sequence is present in the sampled community. On the basis of rRNA sequence comparisons, species-specific DNA- or RNA-hybridization probes subsequently can be designed and used to enumerate the various types of organisms present in an aerosol or biosolids sample. Only recently have some environmental laboratories used rRNA-based molecular techniques in order to identify and characterize human pathogens and commensals aerosolized from the purposeful land application of sewage (Paez-Rubio, 2005). These studies have identified a plethora of microbes associated with human waste and sewage, many of which represent novel genera that previously were not described at the molecular level.

Less than twenty rRNA-based studies of bacterial or fungal bioaerosols have been published to date (included in reference list), with limited numbers of samples and rRNA sequences analyzed. To further investigate the biodiversity of aerosols associated with biosolids and identify potential pathogens liberated during the land application of biosolids, we used broad-range 16S rRNA PCR, cloning, and subsequent sequencing to characterize air samples obtained from liquid impingers immediately circumventing a well defined biosolids application area before, during and after their spreading. This information will be compared to bacteria recovered and identified

using standard clinical laboratory techniques.

MATERIALS AND METHODS

Sample Collection. Bioaerosol samples for genetic analyses were collected using swirling liquid impingers according to accepted methods and manufacturer's specifications (BioSampler, SKC Inc., Eighty Four, PA). The efficiency of the BioSampler filled with 20 ml of water is 79% for 0.3 μm particles, 89% for 0.5 μm particles, 96% for 1 μm particles and 100% for 2 μm particles. Particle-free, autoclaved 0.01 M phosphate-buffer saline (PBS) containing 0.01% Tween 80 (SIGMA, St. Louis, MO) was used as the collection medium in all impingers. 1 ml aliquots were aseptically transferred in a commercial PCR prep hood to DNA free microcentrifuge tubes and were immediately shipped on dry ice to the University of Colorado at Boulder for molecular analysis, following custody protocols approved by the USEPA.

DNA Sample Preparation. A rigorous solvent and grinding DNA extraction protocol was used to process the aerosol samples. Samples were placed in 2 ml microcentrifuge tubes to which 700 μl of Buffer (200 mM NaCl, 200 mM Tris-Cl pH 8.0, 20 mM EDTA, 5% SDS), 500 μl phenol/chloroform and 0.5 g zirconium beads (Biospec Products Inc, Bartlesville, OK) were added. The samples were agitated in a Mini Beadbeater-8 (Biospec Products Inc, Bartlesville, OK) on the highest setting for four minutes and then subjected to centrifugation (13000 rpm) for 5 minutes. The aqueous phase was extracted with phenol/chloroform. DNA was precipitated by addition of NaCl (280 mM final conc.) and 2.5 volumes of ethanol followed by centrifugation (13000 rpm) for 30 minutes. DNA pellets were washed once with 70% ethanol, allowed to dry in a laminar flow hood, and resuspended in 50 μl sterile TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Extracted samples were either placed on ice or stored at –20C before PCR analysis. All DNA extraction and PCR amplification was conducted by Dr. Daniel Frank and Mari Rodriguez in the Phylogentics laboratory of Norman Pace. Although samples were not processed simultaneously, frozen aliquoted reagents were used for DNA extractions and PCR amplifications in order to minimize sample-to-sample variation.

PCR, Cloning, and Sequence Analysis. Small subunit rRNA (SSU rRNA) genes were amplified from DNA samples by PCR with oligonucleotide primers with broad-range specificity for all bacterial SSU rRNA genes: 8F (5'AGAGTTTGATCCTGGCTCAG) and 805R (5'GACTACCAGGGTATCTAAT). Each 30 μl PCR reaction included 3 μl 10x PCR Buffer, 2.5 μl dNTP mix (2.5 mM each dNTP), 1.5μl 50 mM $MgCl_2$, 75 ng of each primer, 1 unit Taq polymerase, and 1 μl genomic DNA lysate (following manufacturers' protocols). PCR reagents from Bioline (Biolase polymerase) and Eppendorf (MasterTaq polymerase) produced indistinguishable results. Thirty cycles of amplification (92C 30 sec., 52C 60 sec., 72C 90 sec.) usually were sufficient to obtain a product of the appropriate length that was visible in ethidium bromide stained agarose gels (Kodak Inc.). Two negative control PCR reactions, one with lysate from control extractions, and the other with sterile H_2O serving as template, were

performed for each set of samples processed in order to assess whether contamination of reagents had occurred. Positive control PCR reactions, which used an environmental genomic DNA sample as template, were also performed for each set of samples processed. For PCR inhibition controls, equal quantities of positive control DNA templates were added to each of two PCR reactions set up in parallel, one of which was supplemented with known quantities of previously sequenced DNA.

DNA fragments were excised from agarose gels (1% agarose gel in tris-borate EDTA) and purified by the QIAquick® gel extraction kit (Qiagen Inc., Valencia, CA). A portion of each PCR product was cloned into the pCR4®-TOPO® vector of the Invitrogen (TOPO® TA Cloning kit following the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). For each clone library that was constructed, 96 transformants were grown overnight at 37C in a 96-well culture plate filled with 1.5 mls of 2xYT medium per well. In order to sequence the inserts of positive transformants, 20 µl of each overnight culture was added to 20 µl of 10 mM Tris-Cl (pH 8.0), heated 10 minutes at 95C, and centrifuged 10 minutes at 4000 rpm in a 96-well plate centrifuge (Eppendorf Inc., Westbury, NY). One µl of culture supernatant was used as template in a 30 µl PCR reaction (38 cycles of the program listed above) with vector specific primers (T7 and T3 sites). Ten µl of each PCR product were first treated with the ExoSap-IT kit (USB Corp, Cleveland, OH) and then subjected to cycle-sequencing with the Big-Dye Terminator kit (Applied Biosystems, Inc., Foster City, CA) following the manufacturers' protocols. Sequencing was performed on a MegaBACE 1000 automated DNA sequencer (Amersham Biosciences, Piscataway, NJ).

Sequence base calling and assembly were performed with proprietary software. Initial microbial species identifications were made by a batch BlastN search (National Center for Biological Information; NCBI) using the client application BlastCl3 (NCBI). SSU rRNA gene sequences were aligned to an existing database of rRNA gene sequences using the computer application ARB. Phylogenetic analyses, including phylogenetic tree estimations, utilized ARB and PAUP*. Statistical analyses were performed using the R software package (v.2.0.1; www.r-project.org). The hypothesis that mean values of species/phylotypes were identical between sample sets was tested by the paired t-test and the paired Wilcoxon rank sum test, at a significance level of $\alpha = 0.01$.

RESULTS

As summarized in Table A-1, broad-range rRNA PCR was successful for 5 of 16 aerosol samples. The identities of microorganisms in the samples were preliminarily determined by querying GeneBank with the wound rRNA sequences using BLAST (basic local alignment search tool). For each sequence analyzed, we defined a "best BLAST hit", which was the GenBank entry with the highest BLAST bit score. In order to cull sequences of poor quality, sequences with lengths less than 500 nucleotides or BLAST bit scores less than 500 were dropped from further analysis. A total of 439 rRNA clones, comprising 5 clone libraries, constituted the final data set.

The distribution of BLAST percent identity scores between the bioaerosol sample sequences and their best BLAST hits provides an estimate of the extent of novel sequence diversity in the clone libraries. Table A-1 shows only the

sample designations for Clone libraries that were constructed and sequenced from each of the mixed-community PCR reactions. The mean percent identity for all sequences was 97% (range 90 – 100%). More than 15% of the clones were identical to previously characterized rRNA sequences. Following alignment of the bioaerosol rRNA data set, sequences were clustered into phylotypes, or relatedness groups defined by > 97% intra-group sequence identity. Although there is no objective criterion for differentiating microbial species on the basis of rRNA sequence similarity, we used a cutoff of 97% identity to define phylotypes because this value provides a conservative estimate of species diversity. By this criterion, the 439 bioaerosol rRNA clones represent 157 phylotypes. Table A-1 also lists the species most closely related (but not necessarily identical) to each of the phylotypes and the number of clones belonging to each phylotype. The prevalences of each phylotype within the specimen set are summarized in Table A-1 as well. Using the most recent genetic library available from GenBank, the most abundant lineages/species were previously uncultured groups of bacteria that could not be classified by current systematic taxonomy (18%), followed by a Beta Proteobacterium (6%), an uncultured L11 bacterium (4%), *Corynebacterium segmentosum* (3%), a *Ralstonia spp*. (3%), *Lactobacillus lactus* SL3 (3%), and a *Sphingomonas* sp. SKJH-30 (2%). Markedly less abundant species also were detected in some of the samples that may be considered as indicators or pathogens associated with sewage sludge, including two *Clostridium* species, several *Lactobacillus* species and a CDC Group DF-3 isolate.

Bacteria identified by DNA sequence analysis listed in Table A-1 were grouped according to their major lineage classifications (family, groups, genus etc). Pie graphs are provided (Figures A-1 to A-6) to show the higher order lineage groupings from each sample where DNA could be extracted for successful PCR. The number of DNA sequences that were amplified in order to make a statistically valid observation from each sample is provided along with the relative abundance of each of the major lineage groupings.

DISCUSSION

In examining the distribution and abundance of the clone libraries compiled from these bioaerosol samples, no trends emerged which suggested that significant amounts of bacteria recovered from aerosols were generated during or after the period when the biosolids were land-applied. This conclusion is based on the following observations: (i) only 5 of the samples could recover DNA in sufficient quantity, and free of inhibition, for successful PCR amplification; (ii) where PCR amplification was successful, biosamplers recovered some types of microorganisms associated with soils regardless of their positions; (iii) while some of the DNA recovered from bioaerosol samples are closely related to potential pathogens or known enteric microorganisms, (e.g. *Clostridium spp*. and *Lactobacillus spp*.), the relative abundance of these sequences was markedly low with respect to other DNA sequences present, and not distributed among the samplers closest to the biosolids application unit; and (iv) no sequences appear in abundance that have been associated with biosolids by conventional culturing techniques, or other recent molecular surveys (WERF, Peccia *et al*., 2004).

In conclusion, broad-range rRNA PCR provided a new perspective to our understanding of aerosol microbiology near biosolids application sites. Given that wind speeds were negligible during this sampling campaign, these results may or may not be indeterminate; indeed they may serve as indication of background in the absence of wind and weathering processes. A molecular survey of microorganisms that are present in the biosolids themselves would increase the yield of this type of study in the context of determining which microbial populations may serve as reliable tracers for bioaerosol aerosolization potential — this level of microbal tracer work is currently being carried out with support from the Water Environment Research Foundation (WERF, Peccia *et al*., 2004). Until recently, characterizing bioaerosols associated with these environments was executed by classical culturing methods, which can be severely limited for their potential to identify and detect a broad range of microorganism; certainly this potential was demonstrated by juxtaposing the clone library and culturing recoveries here. Finally, although this phylogentic study provided a static picture of the bioaerosols collected pre- and post-land spreading, it sets a baseline for future, longitudinal studies that may address the dynamics of airborne microbial ecology associated with biosolids applications.

REFERENCES

Alvarez, A. J., M. P. Buttner, *et al*. (1995). "PCR for bioaerosol monitoring: sensitivity and environmental interference." Applied and Environmental Microbiology **61**: 3639-3644.

Amann, R., W. Ludwig, *et al*. (1995). "Phylogenetic identification and in situ detection of individual microbial cells without cultivation." Microbial Reviews **59**: 143-169.

Angenent, L., S. Kelley, *et al*. (2005). "Molecular identification of potential pathogens in water and air of a hospital therapy pool." Proceedings of the National Academay of Science **10**2: 4860 – 4865.

Boreson, J., A. M. Dillner, *et al.* (2005). "Correlation bioaerosol load with PM2.5 and PM10cf concentrations: a comparison between natural desert and urban fringe aerosols." Atmospheric Environment

Burge, H. A. (1990). "Bioaerosols: Prevalence and health effects in the indoor environment." Journal of Allergy and Clinical Immunology **85**: 687-701.

Burke, J. F., Ed. (1996). PCR: Essential Techniques. Chichester, England, John Wiley and Sons.

Buttner, M. P., K. Willeke, *et al*. (1997). Sampling and Analysis of Airborne Microorganisms. Manual of Environmental Microbiology. C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach and M. V. Walter. Washington D.C., ASM Press**:** 629-640.

Calderon, C., E. Ward, *et al*. (2002). "Detection of airborne fungal spores sampled by rotating-arm and Hirst-type traps using polymerase chain reaction assays." Aerosol Science **33**: 283-296.

Calderon, C., E. Ward, *et al*. (2002). "Detection of airborne fungal spores sampled by rotating-arm and Hirst-type spore traps using polymerase chain reaction assays." Journal of Aerosol Science **33**: 283-296.

Eduard, W. and D. Heederik (1998). "methods for quantitaitve assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments." American Industrial Hygiene Association Journal **59**: 113-127.

Maher, N., H. K. Dillon, *et al*. (2001). "Magnetic bead capture eliminates PCR inhibitors in samples collected form

the airborne environment permitting detection of *Pneumoncystis carinii* DNA." Applied and Environmental Microbiology **67**: 449-452.

Mastorides, S. M., R. L. Oehler, *et al*. (1997). "Detection of airborne Mycobacteria tuberculosis by Air Filtration and polymerase chain reaction." Clinical and Infectios Disease **25**: 756.

Mastorides, S. M., R. L. Oehler, *et al*. (1999). "The detection of airborne Mycobacterium tuberculosis using micropore membrane air sampling and polymerase chain reaction." Chest **115**: 1-25.

McCluskey, R., R. Sandin, *et al*. (1996). "Detection of airborne cytomegalovirus in hospital rooms of immuncompromised patients." Journal of Virological Methods **56**(115-118).

Myatt, T., S. L. Johnston, *et al.* (2004). "Detection of airborne Rhinovirus and its relation to outdoor air supply in office environments." American Journal of Respiratory and Critical Care Medicine **169**: 1187-1190.

NRC (2002). Biosolids Applied to Land- Advancing standards and practices. Washigton, D.C., The National Academies Press.

Nugent, P. G., J. Cornett, *et al*. (1997). "Personal monitoring of exposure to genetically modified microorganisms in bioaerosols: rapid and sensitive detection using PCR." Journal of Aerosol Science **28**: 525-538.

Paez-Rubio, T., E. Viau, *et al*. (2005). "Source bioaerosol concentration and rDNA-based identification of microorganisms aerosolized at a flood irrigation wastewater reuse site." Applied and Environmental Microbiology **71**: In Press.

Palmer, C. J., G. F. Bonilla, *et al*. (1995). "Detection of Legionella species in reclaimed water and air with the EnvironAmp Legionella PCR kit and direct fluorescent antibody staining." Applied and Environmental Microbiology **61**: 407-412.

Pascual, L., S. Perez-Luz, *et al*. (2001). "Detection of *Legionella pneumophila* in boaerosols by polymerase chain reation." Canadian Journal of Microbiology **47**: 341-347.

Peccia, J. (2004) Characterization of Aerosols during the Land Application of Biosolids. Progress Report to the Water Environment Research Foundation, Alexandria, VA.

Pillai, S., K. Widmer, *et al.* (1996). "Ocurrence of Airborne Bacteria and Pathogen Indicators during Land Application of Sewage Sludge." Applied and Environmental Microbiology **62**(1): 296-299.

Sawyer, M., Y. Wu, *et al*. (1994). "Detection of Varicella-Zoster virus DNA in air samples from hospital rooms." The Journal of Infectious Diseases **169**: 91-94.

Sorber, C. and K. Guter (1975). "Health and Hygience aspects fo spray irrigation." American Jounal of Public Health **65**: 47-52.

Stark, K. D. C., J. Nicolet, *et al*. (1998). "Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay." Applied and Environmental Microbiology **64**: 543-548.

Stetzenbach, L. D., M. P. Buttner, *et al.* (2004). "Detection and enumeration of airborne biocontaminants." Current Opinion in Biotechnology **15**: 170-174.

Wakefield, A. E. (1996). "DNA sequences identical to *Pneumocytis carinii* f. sp. *carinii* and *Pneumocytis carinii* f. sp. *hominis* in samples of air spora." Journal of Clinical Microbiology **34**: 1754-1759.

Wan, G., S. Lu, *et al*. (2004). "Polymerase chain reaction used for the detection of airborne Mycobacterium tuberculosis in health care settings." AJIC **32**: 17-22.

Wan, G.-H., S.-C. Lu, *et al.* (2004). "Polymerase chain reaction used for the detection of airbonre *Mycobacterium tuberculosis* in health care settings." American Journal of Infection Control **32**: 17-21.

Williams, R. H., E. Ward, *et al*. (2001). "Methods for integrated air sampling and DNA analysis for detection of airborne fungal spores." Applied and Environmental Microbiology **67**: 2453-2459.

Table A-1. Identity of Clones Based on DNA Similarity of 16s Ribosomal DNA Sequeces Catalogued with GENEBANK

Blast Association	% DNA Similarity	# of Clones in Sample Designation						Bacteria; Lineage
		I0003	I0007	I0028	I0052	I0035	Total	
Uncultured organism clone M8907A05 small subunit ribosomal RNA	97 - 100 (99)	-		26	28	25	79	unclassified; environmental samples.
Uncultured beta proteobacterium clone SM1G08 16S ribosomal RNA	97 - 100 (99)	-	26	-	-	-	26	Proteobacteria; Betaproteobacteria; environmental
Uncultured bacterium clone L11 16S ribosomal RNA gene, partial	99 - 100 (99)	-		5	3	6	16	Bacteria environmental samples.
Corynebacterium segmentosum partial 16S rRNA gene, strain NCTC 934	98 - 99 (98)	14	-	-	-	-	14	Actinobacteria; Actinobacteridae; Actinomycetales;
Uncultured bacterium 16S rRNA gene, clone cD0266	99 - 100 (99)	-	13	-	-	-	13	Bacteria environmental samples.
Ralstonia sp. 1F2 16S ribosomal RNA gene, partial sequence	98 - 100 (99)	-2	12	-	-	-	12	Proteobacteria; Betaproteobacteria; Burkholderiales;
Uncultured proteobacterium clone TAF-B73 16S ribosomal RNA gene,	99	-		9	1	1	11	Proteobacteria; environmental samples.
Lactococcus lactis strain SL3 16S ribosomal RNA gene, complete	99 - 100 (99)	9	-	1	-	-	10	Firmicutes; Lactobacillales; Streptococcaceae;
Sphingomonas sp. SKJH-30 16S ribosomal RNA gene, partial sequence	99 - 100 (99)	-	-	4	4	-	8	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Uncultured bacterium clone TM06 16S ribosomal RNA gene, partial	99 - 100 (99)	-_	1	-	-	7	8	Bacteria environmental samples.
Acinetobacter seohaensis 16S ribosomal RNA gene, partial sequence	98 - 99 (98)	-	-	-	1	6	7	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Uncultured organism clone MC061215 small subunit ribosomal RNA gene,	99	-		2	1	4	7	unclassified; environmental samples.
Staphylococcus epidermidis partial 16S rRNA gene, isolate SLF1	96 - 100 (98)	6	-	-	-	-	6	Firmicutes; Bacillales; Staphylococcus.
Bacteroidetes bacterium LC9 16S ribosomal RNA gene, partial	95 - 96 (95)	-	-	-	5	-	5	Bacteroidetes.
Nocardioides sp. 43/50 16S ribosomal RNA gene, partial sequence	94 - 95 (94)	-_	-	5	-	-	5	Actinobacteria; Actinobacteridae; Actinomycetales;
Streptococcus mitis bv2 16S ribosomal RNA gene, partial sequence	99	5	-	-	-	-	5	Firmicutes; Lactobacillales; Streptococcaceae;
Acidobacteriaceae bacterium TAA43 16S ribosomal RNA gene, partial	96 - 99 (97)	-	2	-	2	-	4	Acidobacteria; Acidobacteriales; Acidobacteriaceae.
Bacterium SM2-6 16S ribosomal RNA gene, complete sequence	98 - 99 (98)	-	-	-	-	4	4	Bacteria.
Lactobacillus plantarum gene for 16S rRNA, partial sequence	98 - 99 (98)	-	-	4	-	-	4	Firmicutes; Lactobacillales; Lactobacillaceae;
Lactococcus lactis subsp. cremoris gene for 16S rRNA, partial	99 - 100 (99)	4	-	-	-	-	4	Firmicutes; Lactobacillales; Streptococcaceae;
Uncultured alpha proteobacterium SBR6alpha8 16S ribosomal RNA gene,	99 - 100 (99)	-	-	-	-	4	4	Proteobacteria; Alphaproteobacteria; environmental
Uncultured bacterium 16S rRNA gene, clone cD02611	96 - 99 (97)	-	4	-	-	-	4	Bacteria; environmental samples.
Uncultured bacterium clone BREC_40 16S ribosomal RNA gene, partial	96 - 98 (97)	4	-	-	-	-	4	Bacteria; environmental samples.
Uncultured Corynebacterium sp. clone ACTINO9C 16S ribosomal RNA	99	4	-	-	-	-	4	Actinobacteria; Actinobacteridae; Actinomycetales;
Agricultural soil bacterium clone SC-I-64, 16S rRNA gene (partial)	91 - 95 (93)	-		-	1	1	3	Bacteria; environmental samples.
Lachnospira pectinoschiza 16S ribosomal RNA gene, partial sequence	99	-	-	3	-	-	3	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;
Leuconostoc pseudomesenteroides DNA for 16S ribosomal RNA, strain	99	-	-	3	-	-	3	Firmicutes; Lactobacillales; Leuconostoc.
Uncultured bacterium clone D8A_5 16S ribosomal RNA gene, partial	99	-1	-	3	-	-	3	Bacteria; environmental samples.
uncultured Bacteroidetes bacterium partial 16S rRNA gene, clone	95 - 96 (95)	-	-	-	3	-	3	Bacteroidetes; environmental samples.
Uncultured Corynebacterium sp. clone ACTINO9B 16S ribosomal RNA	96 - 99 (98)	3	-	-	-	-	3	Actinobacteria; Actinobacteridae; Actinomycetales;
A.calcoaceticus gene for 16S rRNA	100	-	-	-	-	2	2	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Acinetobacter sp. HPC270 16S ribosomal RNA gene, partial sequence	98 - 99 (98)	-	-	-	2	-	2	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Alpha proteobacterium F820 16S ribosomal RNA gene, partial sequence	96 - 97 (96)	-	-	-	2	-	2	Bacteria; Proteobacteria; Alphaproteobacteria.
Bacterium PSD-1-3 16S ribosomal RNA gene, partial sequence	98	-	1	-	1	-	2	Bacteria.
Beta proteobacterium Ellin152 16S ribosomal RNA gene, partial	97	-	-	-	2	-	2	Bacteria; Proteobacteria; Betaproteobacteria.

Table A-1 (continued). Identity of Clones Based on DNA Similarity of 16s Ribosomal DNA Sequeces Catalogued with GENEBANK

Blast Association	% DNA Similarity	# of Clones in Sample Designation						Bacteria; Lineage
		I0003	I0007	I0028	I0052	I0035	Total	
Brachybacterium sp. SKJH-25 16S ribosomal RNA gene, partial	98 - 99 (98)	-	-	2	-	-	2	Actinobacteria; Actinobacteridae; Actinomycetales;
Clostridium lactatifermentans 16S ribosomal RNA gene, partial	92 - 93 (92)	-	-	-	2	-	2	Firmicutes; Clostridia; Clostridiales; Clostridiaceae;
Corynebacterium pseudogenitalium partial 16S rRNA gene, strain	99 - 100 (99)	2	-	-	-	-	2	Actinobacteria; Actinobacteridae; Actinomycetales;
Curtobacterium sp. 1594 16S ribosomal RNA gene, partial sequence	99	-	2	-	-	-	2	Actinobacteria; Actinobacteridae; Actinomycetales;
D.pigrum 16S rRNA gene (partial)	98	2	-	-	-	-	2	Firmicutes; Lactobacillales; Carnobacteriaceae;
Ketogulonogenium vulgarum strain 266-13B small subunit ribosomal	96 - 99 (97)	-	-	2	-	-	2	Proteobacteria; Alphaproteobacteria; Rhodobacterales;
Klebsiella pneumoniae isolate 521 16S ribosomal RNA gene, partial	99 - 100 (99)	-	-	-	-	2	2	Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Klebsiella sp. PN2 gene for 16S rRNA	99	-	-	-	-	2	2	Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Lactobacillus brevis 16S ribosomal RNA gene, partial sequence	99	-	-	-	-	2	2	Firmicutes; Lactobacillales; Lactobacillaceae;
Lactobacillus brevis gene for 16S rRNA, strain:B4101	99	-	-	-	-	2	2	Firmicutes; Lactobacillales; Lactobacillaceae;
Pseudomonas aeruginosa strain PD100 16S ribosomal RNA gene, partial	99	-	2	-	-	-	2	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonas saccharophila 16S ribosomal RNA gene, partial sequence	99	-	2	-	-	-	2	Proteobacteria; Betaproteobacteria; Burkholderiales;
Pseudomonas sp. (strain BKME-9) 16S rRNA gene, partial	99	-	-	-	-	2	2	Bacteria; Proteobacteria.
Pseudomonas sp. pDL01 16S ribosomal RNA gene, partial sequence	99	-	2	-	-	-	2	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Rhizosphere soil bacterium clone RSC-II-81, 16S rRNA gene (partial)	90 - 95 (92)	-	1	-	1	-	2	Bacteria; environmental samples.
Sphingomonas sp. Alpha1-2 16S ribosomal RNA gene, partial sequence	96	-	-	-	-	2	2	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. gene for 16S ribosomal RNA	95 - 96 (95)	-	-	-	2	-	2	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas yunnanensis strain YIM 003 16S ribosomal RNA gene,	99	1	-	-	1	-	2	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Streptococcus pneumoniae strain Kor 145 16S ribosomal RNA gene,	99	2	-	-	-	-	2	Firmicutes; Lactobacillales; Streptococcaceae;
Uncultured bacterium clone DSBR-B020 16S ribosomal RNA gene,	99	-	-	-	-	2	2	Bacteria; environmental samples.
Uncultured bacterium partial 16S rRNA gene, clone SHD-12	98 - 99 (98)	-	-	-	-	2	2	Bacteria; environmental samples.
Uncultured earthworm cast bacterium clone c256 16S ribosomal RNA	96 - 98 (97)	1	1	-	-	-	2	Bacteria; environmental samples.
Uncultured forest soil bacterium clone DUNssu554 16S ribosomal RNA	99	-	-	-	-	2	2	Bacteria; environmental samples.
Uncultured soil bacterium clone G9-1338-5 small subunit ribosomal	98 - 99 (98)	2	-	-	-	-	2	Bacteria; environmental samples.
A.calcoaceticus 16S rRNA gene (DSM30009)	99	1	-	-	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Aeromicrobium erythreum 16S ribosomal RNA gene, partial sequence	96	-	-	1	-	-	1	Actinobacteria; Actinobacteridae; Actinomycetales;
Agricultural soil bacterium clone SC-I-92, 16S rRNA gene (partial)	96	-	1	-	-	-	1	Bacteria; environmental samples.
Agrobacterium sp. NCPPB1650 gene for 16S ribosomal RNA, complete	99	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Rhizobiales;
Alpha proteobacterium 34619 16S ribosomal RNA gene, partial	99	-	-	1	-	-	1	Bacteria; Proteobacteria; Alphaproteobacteria.
Alpha proteobacterium PI_GH2.1.D5 small subunit ribosomal RNA gene,	96	1	-	-	-	-	1	Bacteria; Proteobacteria; Alphaproteobacteria.
Anaerococcus prevotii strain CCUG 41932 16S ribosomal RNA gene,	98	-	-	-	1	-	1	Bacteria; Firmicutes; Clostridia; Clostridiales;
Bacterium RBA-1-13 16S ribosomal RNA gene, partial sequence	97	-	-	-	1	-	1	Bacteria.
Bacterium RBA-1-31 16S ribosomal RNA gene, partial sequence	96	-	1	-	-	-	1	Bacteria.
Bacterium RSD-1-9 16S ribosomal RNA gene, partial sequence	99	1	-	-	-	-	1	Bacteria.
Beta proteobacterium 9c-3 16S ribosomal RNA gene, partial sequence	100	1	-	-	-	-	1	Bacteria; Proteobacteria; Betaproteobacteria.

Table A-1 (continued). Identity of Clones Based on DNA Similarity of 16s Ribosomal DNA Sequeces Catalogued with GENEBANK

Blast Association	% DNA Similarity	# of Clones in Sample Designation						Bacteria; Lineage
		I0003	I0007	I0028	I0052	I0035	Total	
Brevundimonas bacteroides DNA for 16S ribosomal RNA, strain CB7	100	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Caulobacterales;
Brevundimonas diminuta 16S ribosomal RNA gene, partial sequence	98	-	1	-	-	-	1	Proteobacteria; Alphaproteobacteria; Caulobacterales;
Butyrate-producing bacterium SR1/1 16S ribosomal RNA gene, partial	99	-	-	-	1	-	1	Bacteria; Firmicutes; Clostridia; Clostridiales.
Caulobacter sp. DNA for 16S ribosomal RNA, strain FWC38	97	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Caulobacterales;
CDC Group DF-3 16S LMG 11519 ribosomal RNA gene, partial sequence	94	-	-	1	-	-	1	Bacteroidetes; Bacteroidetes (class); Bacteroidales;
Clostridium sp. ArC6 16S ribosomal RNA gene, partial sequence	94	-	-	-	1	-	1	Firmicutes; Clostridia; Clostridiales; Clostridiaceae;
Comamonas testosteroni gene for 16S rRNA, partial sequence	98	-	-	1	-	-	1	Proteobacteria; Betaproteobacteria; Burkholderiales;
Corynebacterium accolens partial 16S rRNA gene, strain CIP104783T	99	1	-	-	-	-	1	Actinobacteria; Actinobacteridae; Actinomycetales;
Cytophagales str. MBIC4147 gene for 16S rRNA, partial sequence	95	-	-	-	1	-	1	Bacteria; Bacteroidetes.
Diaphorobacter sp. PCA039 16S ribosomal RNA gene, partial sequence	100	-	-	1	-	-	1	Proteobacteria; Betaproteobacteria; Burkholderiales;
Earthworm burrow bacterium B33D1 16S ribosomal RNA gene, partial	95	1	-	-	-	-	1	Actinobacteria; Rubrobacteridae; Rubrobacterales;
Flexibacter cf. sancti 16S ribosomal RNA gene, partial sequence	91	-	1	-	-	-	1	Bacteroidetes; Sphingobacteria; Sphingobacteriales;
Glacial ice bacterium G200-C18 16S ribosomal RNA gene, partial	98	1	-	-	-	-	1	Bacteria.
Klebsiella pneumoniae strain 542 16S ribosomal RNA gene, partial	99	-	-	-	-	1	1	Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Lactobacillus brevis gene for 16S rRNA, strain:NRIC 1684	99	-	-	-	-	1	1	Firmicutes; Lactobacillales; Lactobacillaceae;
Lactobacillus parabuchneri gene for 16S ribosomal RNA, partial	99	-	-	1	-	-	1	Firmicutes; Lactobacillales; Lactobacillaceae;
Lactobacillus sp. oral clone CX036 16S ribosomal RNA gene, partial	99	-	-	-	-	1	1	Firmicutes; Lactobacillales; Lactobacillaceae;
Leuconostoc citreum 16S ribosomal RNA gene, partial sequence	99	-	-	1	-	-	1	Firmicutes; Lactobacillales; Leuconostoc.
Leuconostoc pseudomesenteroides 16S ribosomal RNA gene, partial	99	-	-	1	-	-	1	Firmicutes; Lactobacillales; Leuconostoc.
Leuconostoc pseudomesenteroides gene for 16S rRNA, partial	99	-	-	1	-	-	1	Firmicutes; Lactobacillales; Leuconostoc.
Mesorhizobium sp. Ellin189 16S ribosomal RNA gene, partial sequence	98	1	-	-	-	-	1	Proteobacteria; Alphaproteobacteria; Rhizobiales;
Mesorhizobium sp. M01 gene for 16S rRNA, partial sequence	98	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacterium sp. RKT-5 16S ribosomal RNA gene, partial sequence	99	-	-	-	-	1	1	Proteobacteria; Alphaproteobacteria; Rhizobiales;
Mycobacterium sp. Ellin118 16S ribosomal RNA gene, partial sequence	98	-	-	-	1	-	1	Actinobacteria; Actinobacteridae; Actinomycetales;
Nocardioides OS4 16S rRNA	98	-	1	-	-	-	1	Actinobacteria; Actinobacteridae; Actinomycetales;
Nocardioides sp. MWH-CaK6 partial 16S rRNA gene, isolate MWH-CaK6	97	-	1	-	-	-	1	Actinobacteria; Actinobacteridae; Actinomycetales;
Pantoea ananatis partial 16S rRNA gene, strain 0201935	99	-	1	-	-	-	1	Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Paracraurococcus ruber partial 16S rRNA gene, isolate CP2C	96	-	-	1	-	-	1	Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Peptostreptococcaceae bacterium 19gly3 16S ribosomal RNA gene,	94	1	-	-	-	-	1	Firmicutes; Lactobacillales; Leuconostoc.
Potato plant root bacterium clone RC-III-8, 16S rRNA gene (partial)	95	-	-	-	1	-	1	Bacteria; environmental samples.
Pseudomonas aeruginosa 16S ribosomal RNA gene, partial sequence	99	-	1	-	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonas sp. 3B_8 16S ribosomal RNA gene, partial sequence	99	1	-	-	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonas sp. MFY69 16S ribosomal RNA gene, partial sequence	99	1	-	-	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonas sp. TB3-6-I 16S ribosomal RNA gene, partial sequence	100	-	-	1	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonas veronii gene for 16S rRNA, strain:INA06	99	-	-	1	-	-	1	Proteobacteria; Gammaproteobacteria; Pseudomonadales;

Table A-1 (continued). Identity of Clones Based on DNA Similarity of 16s Ribosomal DNA Sequeces Catalogued with GENEBANK

Blast Association	% DNA Similarity	# of Clones in Sample Designation						Bacteria; Lineage
		I0003	I0007	I0028	I0052	I0035	Total	
Rhizosphere soil bacterium clone RSC-II-92, 16S rRNA gene (partial)	97	-	-	-	1	-	1	Bacteria; environmental samples.
Roseburia faecalis strain M6/1 16S ribosomal RNA gene, partial	99	-	-	1	-	-	1	Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;
S.trueperi 16S rRNA gene	96	1	-	-	-	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sejongia jeonii strain AT1047 16S ribosomal RNA gene, partial	97	-	-	-	-	1	1	Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;
Sphingomonas oligophenolica gene for 16S rRNA, partial sequence	98	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. 44/40 16S ribosomal RNA gene, partial sequence	98	-	-	-	-	1	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. ATCC 53159 16S ribosomal RNA gene, partial	98	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. CJ-5 partial 16S rRNA gene, isolate CJ-5	97	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. KIN163 16S ribosomal RNA gene, partial sequence	97	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. KIN169 16S ribosomal RNA gene, partial sequence	96	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. SAFR-028 16S ribosomal RNA gene, partial sequence	97	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. SRS2 16S rRNA gene, strain SRS2	96	-	1	-	-	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonas sp. YT gene for 16S rRNA	97	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Spirosoma sp. 2.8 partial 16S rRNA gene, strain 2.8	93	1	-	-	-	-	1	Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Staphylococcus epidermidis AB111112 16S ribosomal RNA gene, partial	99	1	-	-	-	-	1	Firmicutes; Bacillales; Staphylococcus.
Uncultured alpha proteobacterium 16S rRNA gene, clone BIrii13	97	1	-	-	-	-	1	Proteobacteria; Alphaproteobacteria; environmental
Uncultured alpha proteobacterium clone ALPHA5C 16S ribosomal RNA	93	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; environmental
Uncultured alpha proteobacterium clone S1-10-CL6 16S ribosomal RNA	99	-	-	-	1	-	1	Proteobacteria; Alphaproteobacteria; environmental
Uncultured alpha proteobacterium SBR8alpha5 16S ribosomal RNA gene,	98	1	-	-	-	-	1	Proteobacteria; Alphaproteobacteria; environmental
Uncultured bacterium clone 1974b-28 16S ribosomal RNA gene, partial	96	1	-	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone 33-FL34B99 16S ribosomal RNA gene,	98	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone 76 16S ribosomal RNA gene, partial	98	-	-	-	-	1	1	Bacteria; environmental samples.
Uncultured bacterium clone A4 16S ribosomal RNA gene, partial	97	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone ABLCf6 16S ribosomal RNA gene, partial	99	-	-	1	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone B8 16S small subunit ribosomal RNA gene,	99	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone D3 16S ribosomal RNA gene, partial	98	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone E9 16S small subunit ribosomal RNA gene,	97	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone FB33-24 16S ribosomal RNA gene, partial	98	1	-	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone FBP249 16S ribosomal RNA gene, partial	90	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone LJ3 16S ribosomal RNA gene, partial	96	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone LO13.11 16S ribosomal RNA gene, partial	98	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone mek62a01 16S ribosomal RNA gene, partial	96	-	-	1	-	-	1	Bacteria; environmental samples.
Uncultured bacterium clone O-CF-31 16S ribosomal RNA gene, partial	96	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone REC6M_59 16S ribosomal RNA gene, partial	99	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone S1-1-CL4 16S ribosomal RNA gene, partial	94	1	-	-	-	-	1	Bacteria; environmental samples.

Table A-1 (continued). Identity of Clones Based on DNA Similarity of 16s Ribosomal DNA Sequeces Catalogued with GENEBANK

Blast Association	% DNA Similarity	# of Clones in Sample Designation						Bacteria; Lineage
		I0003	I0007	I0028	I0052	I0035	Total	
Uncultured bacterium clone synarJM02 16S ribosomal RNA gene,	96	-	-	-	1	-	1	Bacteria; environmental samples.
Uncultured bacterium clone ZEBRA_19 16S ribosomal RNA gene, partial	94	-	-	1	-	-	1	Bacteria; environmental samples.
Uncultured bacterium partial 16S rRNA gene, clone MCS2/83	94	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured bacterium partial 16S rRNA gene, clone Sta0-39	99	1	-	-	-	-	1	Bacteria; environmental samples.
Uncultured Bacteroides sp. clone TNHu1-10 16S ribosomal RNA gene,	92	-	-	-	-	1	1	Bacteroidetes; Bacteroidetes (class); Bacteroidales;
Uncultured beta proteobacterium clone FTL217 16S ribosomal RNA	99	1	-	-	-	-	1	Proteobacteria; Betaproteobacteria; environmental
Uncultured eubacterium 16S rRNA gene, clone LKB47	99	-	-	-	1	-	1	Bacteria; environmental samples.
uncultured eubacterium WD208 partial 16S rRNA gene, clone WD208	95	-	1	-	-	-	1	Bacteria; environmental samples.
uncultured eubacterium WD286 partial 16S rRNA gene, clone WD286	98	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured forest soil bacterium clone DUNssu642 16S ribosomal RNA	99	-	-	1	-	-	1	Bacteria; environmental samples.
Uncultured organism clone M8907G12 small subunit ribosomal RNA	95	-	-	-	1	-	1	unclassified; environmental samples.
Uncultured soil bacterium clone 359 small subunit ribosomal RNA	95	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured soil bacterium clone 5-1 small subunit ribosomal RNA	94	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured soil bacterium clone 749-2 16S ribosomal RNA gene,	98	1	-	-	-	-	1	Bacteria; environmental samples.
Uncultured soil bacterium clone Tc124-C11 16S ribosomal RNA gene,	95	-	1	-	-	-	1	Bacteria; environmental samples.
Uncultured yard-trimming-compost bacterium clone S-19 16S ribosomal	94	-	-	-	1	-	1	Bacteria; environmental samples.
Unidentified bacterium clone W4-B50 16S ribosomal RNA gene, partial	95	-	-	-	-	1	1	Bacteria; environmental samples.
Zoogloea sp. AI-20 16S ribosomal RNA gene, partial sequence	98	-	-	-	1	-	1	Proteobacteria; Betaproteobacteria; Rhodocyclales;
		81	90	86	95	87	439	

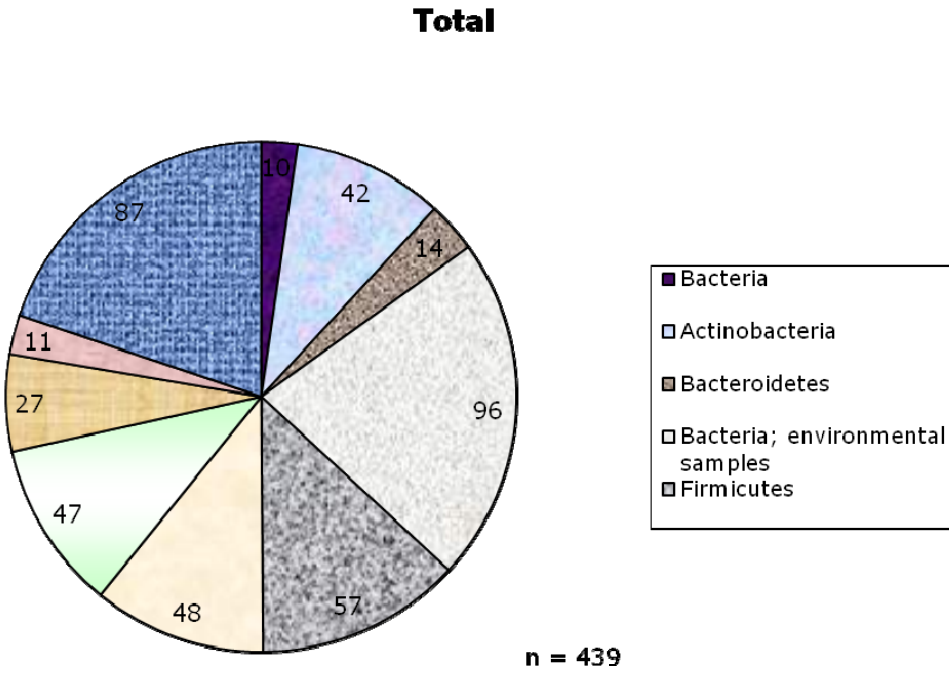


Figure A-1.

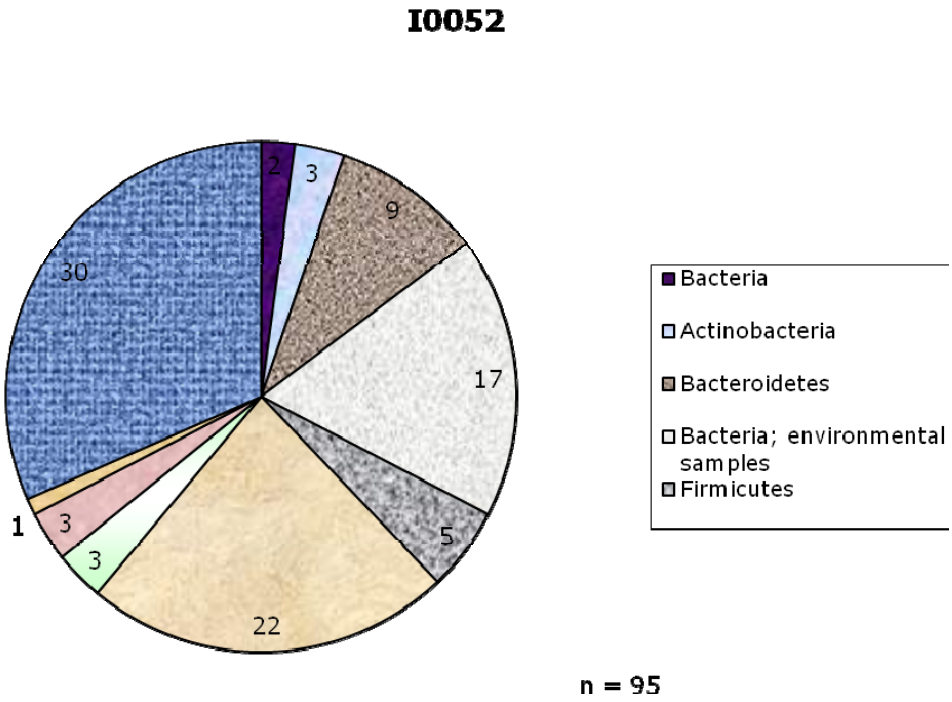


Figure A-2.

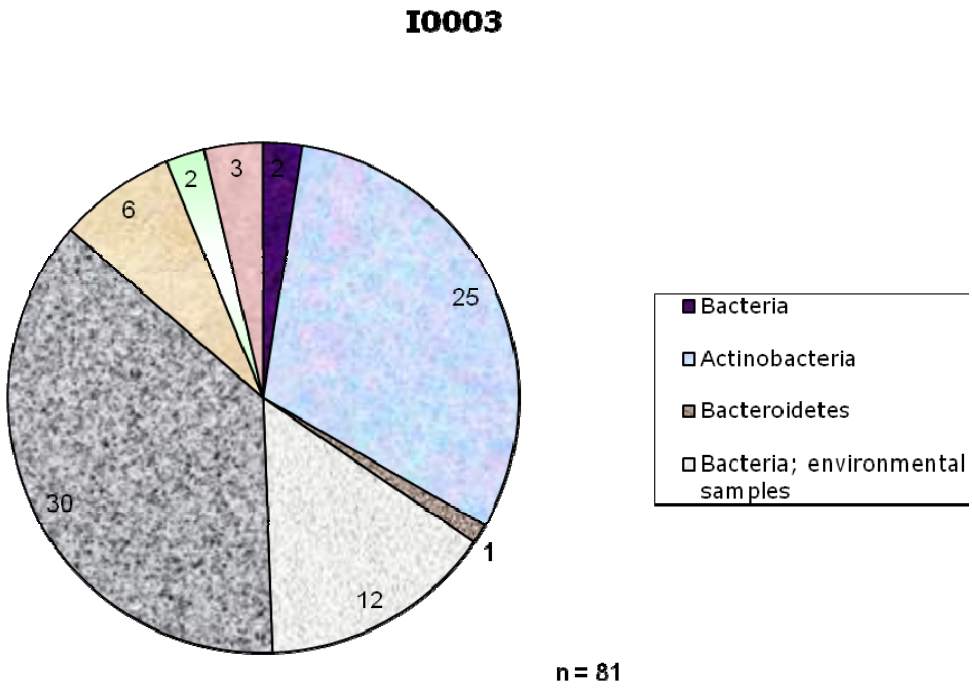


Figure A-3.

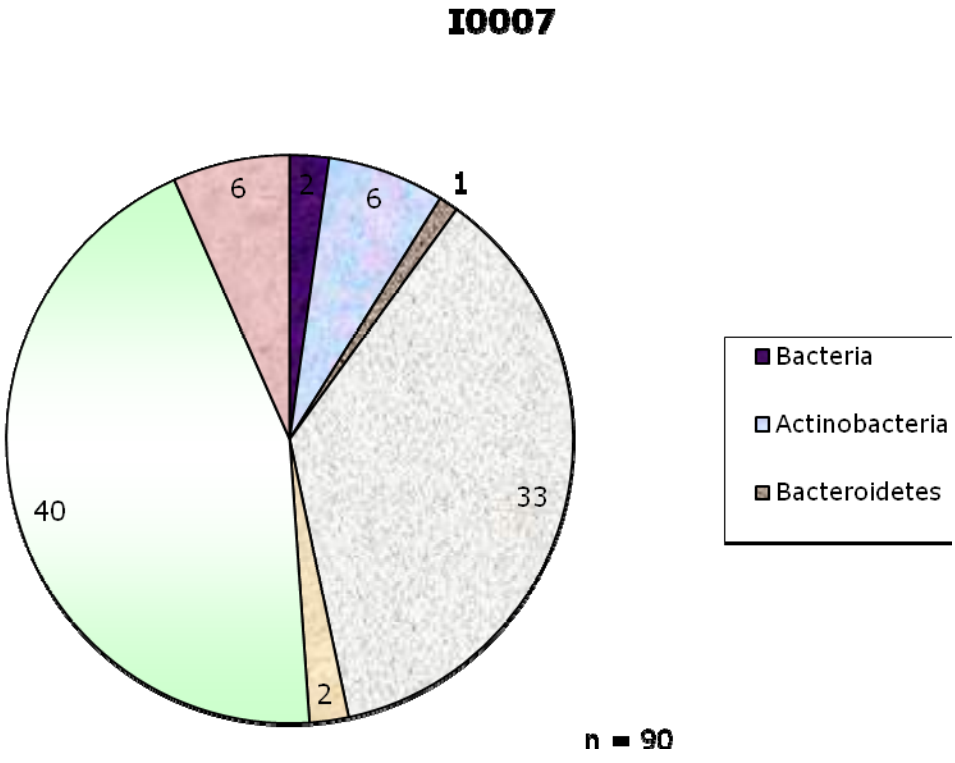


Figure A-4.

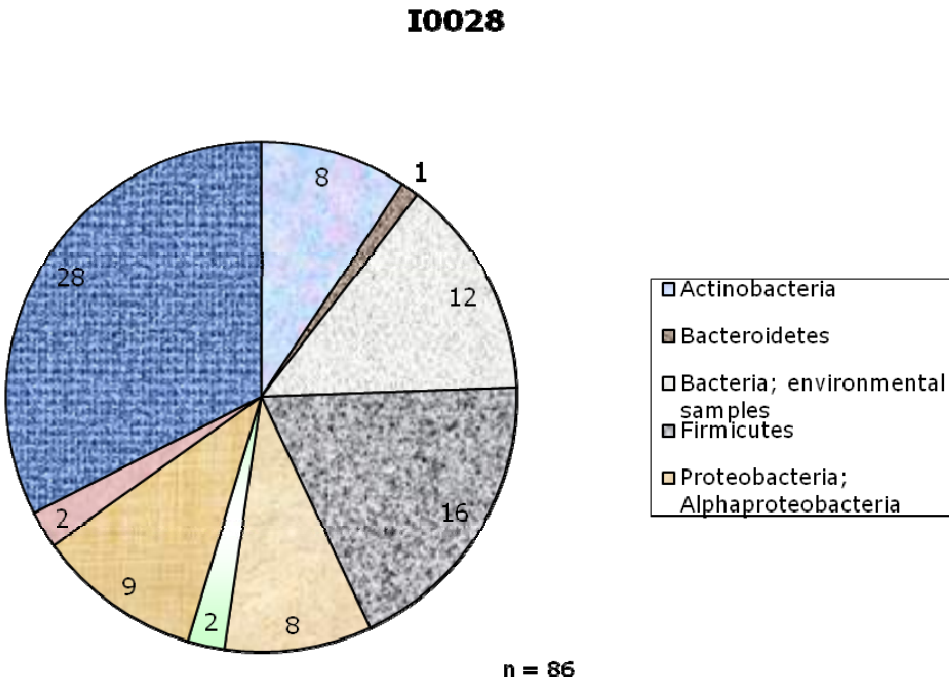


Figure A-5.

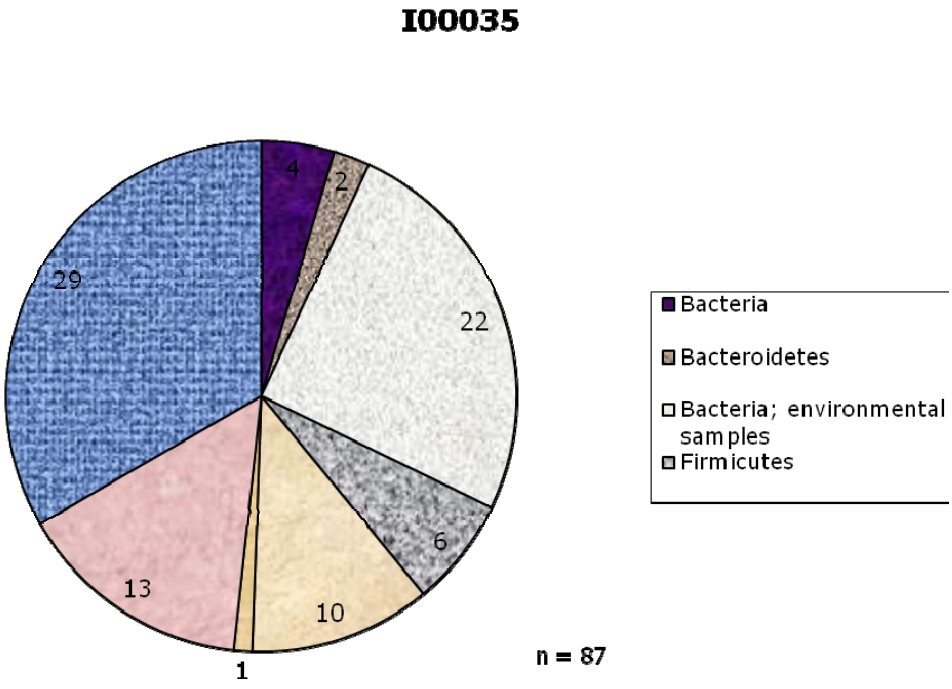


Figure A-6.

APPENDIX B

Parallel Sampling Approaches and Analysis of Impinger Samples Collected During the NC Biosolids Land Application Study – Dr. Ian Pepper, University of Arizona

North Carolina Field Aerosol Sampling
Dates: 9-27-04 – 9-30-04

Objectives:

1. To collect aerosol samples downwind of a land application of biosolids site located in Salisbury, NC and analyze for microbial content.

Results:

Aerosol samples were collected in multiples of 6 from approximately 2 m downwind from the site perimeter, constituting a sampling array. This was conducted 3 times during biosolids land application. In addition a set of 6 samples were collected 2 m from the site perimeter prior to biosolids application, termed background samples (BG). Samples were analyzed for the presence of heterotrophic plate count bacteria (HPC), total coliforms, *Escherichia coli*, *Clostridium perfringens*, and coliphage. In addition, a 5 ml aliquot from each sample was set aside for pathogenic virus analysis. To increase sensitivity, the 5 ml from the 6 samples collected during each separate array were combined and concentrated using Centriprep 50 concentrators to generate 1 sample from the array of 6. The final volume (< 1.0 ml) was used in enteric virus viability assays (cell culture – BGMK cells, incubation 14 d, two passages) and used for reverse transcriptase polymerase chain reaction (RTPCR) for the detection of enterovirus, hepatitis A virus, and norovirus ribonucleic acid (RNA). Finally in addition to aerosol samples a sub-sample of Class B biosolids was analyzed for the presence of the previously mentioned microbial indicators and pathogens.

Overall, aerosol samples were negative for the presence of microbial indicators and microbial pathogens. Aerosolized heterotrophic plate count bacteria densities were approximately 7.27×10^3 during biosolids land application, while during background sample collection, HPC concentrations were approximately 3.18×10^3 (Table B-1). HPC concentrations during background and downwind sample collections were similar, possibly due to the soil moisture and ambient relative humidity levels, which led to overall low levels of aerosolized HPC bacteria from both biosolids and soil. In addition, the location of the sample placement was such that the biosolids applicator began application approximately 10 m upwind of the sampler locations, even though the sampler location was 2 m from the edge of the field perimeter. In addition biosolids application proceeded from right to left in a circular fashion relative to the sampler location. Upon proceeding to the opposite end of the circle (180 º) the samplers were no longer in line of sight of the biosolids application, due to the presence of an uneven field in which the center of the field was approximately 2 - 4 m above the edges of the field, forming a convex field. As such exposure to biosolids only took place for the brief instant that the applicator was in range of the samplers, thus limiting aerosol concentrations.

Table B-1. Aerosol microbial concentrations detected during September 29 and September 30, 2004.
* NA – Refers to no data collected

										RTPCR			Cell Culture		
	Sample	Ambient Climate Conditions								Virus Presence/Absence					
Sample	Placement	Temp	RH	WS	HPC	Total Coliform	*E. coli*	*C. perfringens*	Coliphage	Enterovirus	HAV	Norovirus	Enterovirus	HAV	Norovirus
		C	%	ms^{-1}			CFU, MPN, PFU m-3								
1	BG	NA	NA	NA	5.60E+03	0	0	0	0						
2	BG				1.87E+03	0	0	0	0						
3	BG				3.47E+03	0	0	0	0						
4	BG				1.33E+03	0	0	0	0						
5	BG				4.27E+03	0	0	0	0						
6	BG				2.40E+03	0	0	0	0	Neg	Neg	Neg	Neg	Neg	Neg
Avg					**3.16E+03**										
7	DW	20.2	88.0	1.0	6.13E+03	0	0	0	0						
8	DW				5.60E+03	0	0	0	0						
9	DW				8.00E+03	0	0	0	0						
10	DW				9.33E+03	0	0	0	0						
11	DW				9.33E+03	0	0	0	0						
12	DW				8.27E+03	0	0	0	0	Neg	Neg	Neg	Neg	Neg	Neg
Avg					**7.78E+03**										
13	DW	22.0	52.0	0.8	1.22E+04	0	0	0	0						
14	DW				5.65E+03	0	0	0	0						
15	DW				8.78E+03	0	0	0	0						
16	DW				4.71E+03	0	0	0	0						
17	DW				7.22E+03	0	0	0	0						
18	DW				5.02E+03	0	0	0	0	Neg	Neg	Neg	Neg	Neg	Neg
Avg					**7.27E+03**										
19	DW	29.4	39.0	0.0	7.82E+03	0	0	0	0						
20	DW				4.27E+03	0	0	0	0						
21	DW				7.47E+03	0	0	0	0						
22	DW				4.98E+03	0	0	0	0						
23	DW				3.56E+03	0	0	0	0						
24	DW				6.40E+03	0	0	0	0	Neg	Neg	Neg	Neg	Neg	Neg
Avg					**5.75E+03**										

Neg – Refers to negative results, none detected
CFU – Colony forming unit
MPN – Most probable number
PFU – Plaque forming unit
Temp – Temperature
RH – Relative Humidity
WS – Windspeed
BG – Background aerosol sample
DW – Downwind aerosol sample

APPENDIX C

Endotoxin Sampling During a Post-Spring Cutting Event at the NC Biosolids Land Application Study Site – Dr. Edwin Barth, EPA/NRMRL

Evaluation of Airborne Endotoxin Concentrations Associated with Management of a Crop Grown on Applied Biosolids

E. BARTH*, R. HERRMANN, T. DAHLING, R. BRENNER, S. WRIGHT and P. CLARK
National Risk Management Research Laboratory, Office of Research and Development, USEPA, Cincinnati, OH

ABSTRACT: Public health concerns have been expressed regarding inhalation exposure associated with the application of biosolids on cropland, which is due to the potential aerosolization of microorganisms, cell wall products, volatile chemicals, and nuisance odors. Endotoxin is a component of the cell walls of Gram-negative bacteria and is likely present in many biosolids. The application of biosolids to cropland may result in an immediate exposure or a delayed exposure to these microbial agents, such as when the crops are harvested. Upwind and downwind airborne concentrations of endotoxin were compared among and within two adjacent established hayfields, one with and one without previously applied biosolids, during grass raking and bailing activities. The mean downwind concentration of airborne endotoxin was significantly higher than the mean upwind concentration at the site where biosolids had been previously applied. The mean downwind concentration of endotoxin was not significantly different than the mean upwind concentration at the control field where biosolids had not previously been applied. When comparing the mean concentrations of airborne endotoxin among the sites, significant main effects were noticed for wind direction and field type, and an interactive effect was noticed for direction and field type. It is not known if the increased mean concentration of endotoxin associated with the downwind air samples at the applied biosolids field were due to the residual biosolids that were previously applied or due to endotoxin associated with plant material. The results apply to this investigation only since there was no treatment replication of each type of field. The downwind endotoxin concentrations observed during the raking and bailing activities were lower than various health effects criteria that have been recommended for airborne endotoxin.

INTRODUCTION

ENDOTOXIN is a term associated with the toxic characteristics of the outer membrane of Gram-negative bacteria [1], specifically the fragments of the Gram-negative cell wall that contain lipopolysaccharides [2]. Lipopolysaccharides are essential for the physical organization and function of the outer membrane, and thus for bacterial growth and multiplication [3]. Endotoxin consists of a family of molecules called lipopolysaccharide (LPS). The LPS contains a lipid region (lipid A), and a long covalently linked heteropolysaccharide. The polysaccharide portion is divided into a core portion and the O-specific chain [2,4]. Endotoxin is present in the environment as whole cells, large membrane fragments, or macromolecular aggregates of about one million Daltons [5].

The multiple biological activities associated with endotoxin reside in the lipid A component [6,7]. The biological activity of endotoxin is not dependent on bacterial viability [8]. Human inhalation studies or worker exposure cases involving endotoxin have shown adverse physiological and symptomatic respiratory responses [9,10]. Inhalation of the components of bioaerosols may result in several allergenic-type reactions or lung diseases, such as bronchitis, reactive airway disease, organic dust toxic syndrome (ODTS), and hypersensitivity pneumonitis (HP) [11]. There is debate whether early childhood exposure to endotoxin is positively or negatively associated with the onset or severity of asthma [12,13].

Endotoxin is released into the environment after bacterial cell lysis or during active cell growth [14]. Since bacteria, fungi, and endotoxin may be associated with biosolids, there is an inhalation concern with these bioaerosol components both during and after biosolids land application. Bacteria in biosolids may survive for long periods of time, depending upon the method of

*Author to whom correspondence should be addressed.
E-mail: barth.ed@epa.gov

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management and environmental conditions [15,16,17]. Elevated levels (above background) of endotoxin were associated with sites receiving biosolids application with a mechanical slinger [18]. There is no published study regarding airborne endotoxin concentrations during subsequent crop management activities.

The primary objective of this study was to determine if a statistically significant difference existed between the mean upwind and mean downwind airborne concentrations of endotoxin, during grass raking and bailing activities among and within two proximal hayfields (grass), with one of the sites having been previously treated with biosolids as a soil amendment.

MATERIALS AND METHODS

The sampling approach for this study involved two separate sampling events for aerosolized endotoxin. One sampling event occurred at an established hayfield that did not receive any biosolids application (control field). The other sampling event occurred at an established hayfield that had received surface applied biosolids (application field). Anaerobically digested biosolids were similar to Class B biosolids, but were pre-treated with a limited amount of lime to ensure a viable microbial population was present for monitoring purposes. The biosolids contained approximately 10^9 colony forming units per gram-dry weight (CFU gdw^{-1}) total coliforms with a solids content of 22%. The biosolids were applied to the application field within a 100 m diameter area, approximately nine months earlier, and were applied to the surface via a hopper truck with a mechanical slinger at a rate of 10 dry tons per acre.

Each of the two sampling events occurred during separate grass raking and bailing activities (3 dry-days after grass cutting) for approximately 60 minutes. For each sampling event, five upwind and five downwind stations (containing two endotoxin sampling devices each) were placed along parallel lines, perpendicular to the prevailing wind direction, as shown in Figure 1. The exact orientation of the zones was determined based upon the weather station wind direction data collected by a Davis Instruments Weather Monitor II weather station (Hayward, California). The samplers were oriented around a 40 m × 40 m monitoring area (within a 40 m × 80 m area that had been cut). For the application site, the monitoring area was within the original 100 m diameter biosolids application area. Five upwind and five downwind sampling stations, each containing two endotoxin samplers, were located 10 m apart from each other

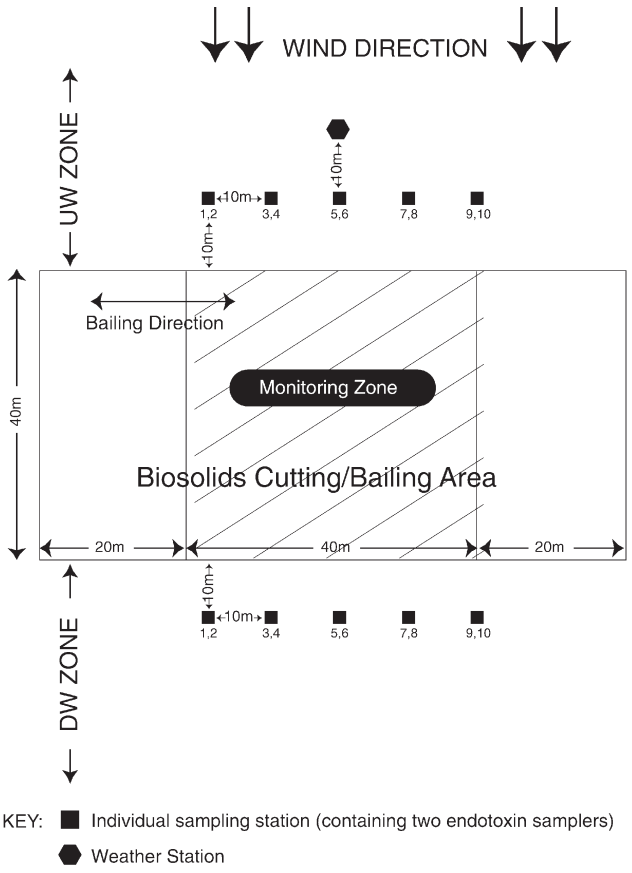

Figure 1. *Sampling station orientation for both control and application fields.*

within the respective zone. For both the upwind and downwind zones, the distance from the samplers to the corresponding external edge of the biosolids raking and bailing area was 10 m. The bailing machine was operated parallel to the sampler lines. After each pass, the raking and bailing equipment (two distinct farm machine vehicles in series) temporarily left the sampling zone, turned around, and performed another pass in the opposite direction (endotoxin samplers continued to operate during the turn-around). The samples were collected near the personal breathing zone (PBZ) height at 1.5 m above the ground surface by mounting the endotoxin samplers on portable tripods. The weather station was placed 20 m upwind and on the mid-line of the upwind sampling line.

The control field (of the same size) contained the same grass cover as the biosolids field. It was located approximately 400–500 m from the biosolids field. To reduce field variation, one initial fertilizer application was applied to the control field within three months of the demonstration, since the application site received nitrogen loading from the applied biosolids.

Prolonged wind direction changes of more than 45 degrees, for longer than two minutes, or any strong wind gust (greater than 15 MPH for at least two minutes), or any precipitation event would immediately halt sample collection activities until they subsided. The bailing equipment was instructed to shut-down at this time as well. If the sampling was shut down for more than 30 continuous minutes, the sampling event would have been considered to be invalid.

Various sampling methods for collecting airborne endotoxin have been used in occupational settings [9, 19]. There are several variables which will possibly influence the endotoxin concentration collected in air samples, such as filter type, extractant fluid, and sample preservation time [20]. The sampling method used involved the use of commercially available 37-mm cassette filter (0.45 μm polycarbonate filters) assemblies (Aerotech Laboratories, Phoenix, AZ), which were manufactured to be endotoxin-free. Two cassettes were mounted to each tripod for each of the sampling stations. The cassettes were separately attached to a vacuum pump (GAST Manufacturing, Benton Harbor, MI). The desired collection flow rate for the cassette assemblies was 4.0 L min^{-1}. Each air collection pump was calibrated pre- and post-sampling in the field immediately before and after each sampling event with a primary standard calibrator (Gillian Model 2 primary standard pump calibrator). Any pre- and post-flow rates that differed by more than 10% were not used in subsequent data calculations.

After each sampling event, the cassettes were capped, placed in plastic bags, and then placed into an iced cooler for transport back to the analytical laboratory within 8 hours. After arrival at the laboratory, cassettes were opened, cassette filters were aseptically removed, and then the filters were placed into a pyrogen free 50 ml centrifuge tube containing 6 ml of pyrogen free water. The 50 ml centrifuge tube was capped and shaken on a mechanical shaker for one hour to complete the extraction procedure for endotoxin.

After the endotoxin was extracted, it was assayed using the Kinetic-QCL method [21]. The field samples were mixed with a substrate, placed in the kinetic reader, and monitored (time) for the appearance of a yellow color. A standard dilution curve ranging from 0.005–50 Endotoxin Units (EU) ml^{-1}, using a control standard endotoxin (CSE), was prepared during the assay. A positive product control spike (PPC) for each dilution was incorporated into the assay to determine recovery (–50%–200%). The solutions were delivered onto a 96-well microplate, which was then inserted into the BioWhittaker Kinetic QCL reader.

Descriptive and inferential statistical methods were used on the collected data. Two approaches were considered for the analysis. In the first approach, the sites were considered independent of each other. Inferential statistics included the parametric student t-test assuming normality of the data distribution. The null hypothesis was that there is no statistically significant difference in airborne endotoxin concentration between the mean upwind zone concentration (10 samples) and mean downwind zone concentration (10 samples) of endotoxin, for each sampling event. The second approach analyzed the data as a completely randomized design with a two-way treatment structure (2×2), wind direction (upwind, downwind) and field type (control, biosolids) using ANOVA (PROC GLM Procedure). The *t*-test and ANOVA analyses were performed using SAS [22].

RESULTS AND DISCUSSION

The airborne concentrations of endotoxin at each identified station, and the mean concentrations for each trial, are provided in Table 1. The values in Table 1 were adjusted for concentrations of endotoxin detected in the field and blank samples. Interactive box plots (field type by wind direction) of the endotoxin data is presented in Figure 2, showing a potential outlier sample value for each trial. None of the environmental conditions that would have invalidated the results were encountered, so the sample collection effort was considered valid.

Table 1. Concentration of Airborne Endotoxin at Individual Sampling Stations (EU m^{-3}).

Field	Zone	1	2	3	4	5	6	7	8	9	10	Mean
Control	Upwind	11.3	6.1	4.8	3.1	8.6	9.5	8.7	8.7**	7.9	10.6	7.8
Control	Downwind	11.6	38.1*	14.9**	6.3	9.7	15.6	16.1	7.4	14.2	11.9	14.5
Application	Upwind	7.7	2.9	2.0	2.5	0.3	0.4	0.0	2.5**	1.5	2.0	2.1
Application	Downwind	24.3	27.1	38.2**	28.9	43.5	44.8	42.7	34.2	39.9	38.3	36.0

*Represent potential outlier data.
**Stations greater than 10% difference in pre/post flow calibration.

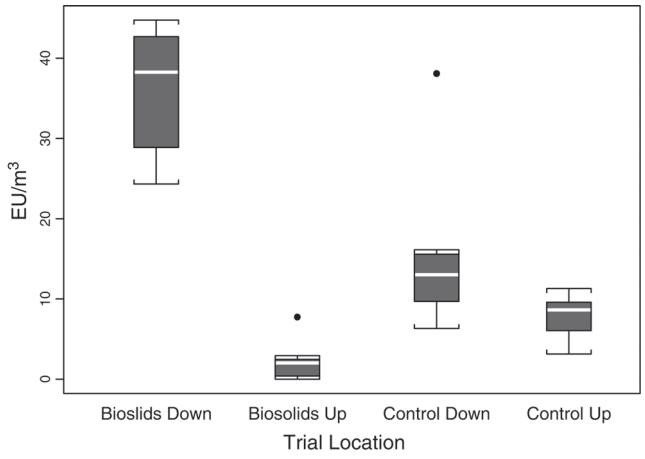

Figure 2. *Boxplots of upwind and downwind endotoxin concentrations for control and biosolids application trials.*

For relative comparisons, the mean airborne endotoxin concentrations observed for each trial were greater than some of the other published background range levels detected in outside environments that vary from 0.0005–0.74 EU m^{-3} in outdoor environments in Germany to 2.0–3.8 EU m^{-3} for outdoor sites in the United States [23,24,18]. The levels observed were lower than the mean concentration of 114 EU m^{-3} observed within 10 m downwind of a limited number of biosolids application sites in the southwestern United States, but in the range of the mean concentration of 6 EU m^{-3} observed further downwind on these sites.

The mean airborne endotoxin concentrations observed for each trial were less than published occupation exposure levels, and less than the large range of other published human exposure criteria for endotoxin. Inhalation of endotoxin in concentrations as low as 4–15 ng m^{-3} (40–150 EU m^{-3}) has been associated with acute and chronic airway inflammation and lung function decrements [23]. The International Committee on Occupational Health (ICOH) Committee on Organic Dust observed toxic pneumonitis at endotoxin levels of 200 ng m^{-3} (2000 EU m^{-3}), systemic reactions at 100 ng m^{-3} (1000 EU m^{-3}), and airway inflammation at 10 ng m^{-3} (100 EU m^{-3}) [25]. Experimental studies of human exposure to cotton dust and field studies suggest an endotoxin threshold for acute airflow obstruction in the range of 45 to 330 EU m^{-3} [26]. The ACGIH has recommended an indoor endotoxin concentration less than 30 to 100 times the ambient (outdoor) concentration [26]. The Dutch Expert Committee on Occupational Standards of the National Health Council has proposed a health-based recommended limit value of 4.5 ng m^{-3} (0.45 EU m^{-3}) over an eight-hour exposure period [27]. The NIOSH Recommended Exposure Limit (REL) for airborne metalworking particulates, that may contain endotoxin from recirculated fluids, is limited to 0.4 mg m^{-3} for thoracic particle mass (0.5 mg m^{-3} total particulate mass) [28].

The mean concentration of downwind airborne endotoxin samples was significantly higher than upwind concentration mean during grass raking and bailing operations within the application trial field where biosolids had previously been applied. The mean upwind and downwind concentrations were not statistically significantly different within the control trial field where biosolids had not previously been applied. It was not determined if the increased concentrations of endotoxin in the downwind air samples at the biosolids application field were due to the biosolids residual or due to plant material grown on the field. Even though the control field did receive fertilizer, the density of plant material appeared to be visually higher on the biosolids application site, though any type of measurement for this property was not performed.

The mean concentration of the downwind air samples at the application site was statistically different than the other three means (upwind control, downwind control, upwind application). However, the downwind control trial mean was higher than the upwind means for both trials (before multiple comparison adjustment) and higher than the upwind application trial site mean even after adjustment (via Scheffe's approach). ANOVA analysis between the two sites (four groups) indicated that there were statistically significant main effects among the sites in wind direction and field type; there was also a statistically significant interaction effect with wind direction and field type. The residuals from the ANOVA are not normal, but also are not skewed, so a transformation (such as the logarithmic) was not useful to normalize the distributions. However, the box plots did identify possible influential outliers. Suspected outliers made no difference in interpretation, and after removing these outliers, the relationship between the endotoxin concentration and the type of field and sampler location is strengthened. After removal of the outliers, the distribution of the residuals becomes normal, indicating that the two outliers contributed to the non-normality of the original data distribution but were not influential. The results apply to this investigation only since there was no treatment replication of each type of field.

CONCLUSIONS

The mean downwind concentration of airborne endotoxin associated with raking and bailing of grass was significantly higher than the mean upwind concentration at a specific hayfield site where biosolids had been applied approximately nine months prior to the sampling event. It was not determined if the increased mean concentration of endotoxin in the downwind air samples at the biosolids application field were due to biosolids residuals or due to plant material grown on the field. In contrast, the mean downwind concentration of airborne endotoxin for the same activities at a close proximity site (control site) that did not receive biosolids application was not significantly higher than the mean upwind concentration.

ACKNOWLEDGEMENTS

The cooperation of several individuals from the Agricultural Research Station in Salisbury, NC is greatly appreciated. Larry Wetzel of the USEPA participated in the sample collection effort.

REFERENCES

1. Rietschel, E. and H. Brade. "Bacterial Endotoxin", *Sci. Amer.* Vol. 267, No. 2, 1992, pp. 54–61.
2. Jacobs, R. D. Heederik, J. Douwes, and U. Zahringer. "Endotoxin Structure", *Int. J. Occ. Health.* Vol. 3(1 Suppl), 1997, pp. S6–S7.
3. Moran, A. "Structure-Bioactivity Relationship of Bacterial Endotoxins", *J. Toxic.—Toxin Rev.,* Vol. 14, No.1, 1995, pp. 47–83.
4. Schletter, J., H. Heine, A. Ulmer, E. Rietschel. "Molecular Mechanisms of Endotoxin Activity". *Arch. of Microb.* Vol. 164, 1995, pp. 383–389.
5. Milton, D. 1995. Endotoxin. *Bioaerosols,* H. Burge, editor, CRC Press, Boca Raton, FL.
6. Ulmer, A. "Biochemistry and Cell Biology of Endotoxins. Human Challenge Studies with Endotoxins" *Int. J of Occ. Health,* Vol. 3, No. 1(1), 1997, pp. S8–S15.
7. Shiba, T. and S. Kusumoto. 1984. Chemical Synthesis and Biological Activity of Lipid A Analogs. *Handbook of Endotoxin,* Vol. 1, *Chemistry of Endotoxin,* E. Rietschel, editor, Elsevier Science Publishers.
8. Jacobs, R. "Airborne Endotoxins: An Association with Occupational Lung Disease". *App. Ind. Hyg.,* Vol. 4, No. 2, 1989, pp. 50–56.
9. Olenchock, S. 1997. Airborne Endotoxin. *Manual of Environmental Microbiology,* C. Hurst, editor, ASM press, Washington, DC.
10. Rylander, R., B. Rake, J. Fischer, and I. Helander. "Pulmonary Function and Symptoms After Inhalation of Endotoxin", *Amer. Rev. Resp. Dis.,* Vol. 140, No. 4, 1989, pp. 981–986.
11. Harber, P., M. Schenker, and J. Balmes. 1996. *Occupational and Environmental Respiratory Diseases,* Mosby Publishing, 1st edition, St. Louis, MI.
12. Salem, M., Y. Li, B. Langholz and F. Gillialand. "Early-Life Environmental Risk Factors for Asthma" *Env. Health Persp.,* Vol. 112, No.6, 2004, pp. 760–765.
13. Braun-Fahrlander, C., U. Herz, W. Eder, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Buff, R. Schierl, H. Renz, D. Nowak, and E. von Mutius. "Environmental Exposure to Endotoxin and its Relation to Asthma School Aged Children". *New Eng. J. Med.* Vol. 347, No.12, 2002, pp. 869–877.
14. Olenchock, S. "Health Effects of Biological Agents: The Role of Endotoxins", *App. Occ. Env.Hyg.,* Vol. 9, No. 1, 1994, pp. 62–64.
15. Gibbs, R., C. Hu, G. Ho, and I. Unkovich. "Regrowth of Faecal Coliforms and Salmonella in Stored Biosolids and Soil Amended with Biosolids", *Wat. Sci. Tech.,* Vol. 35, No.11/12, 1997, pp. 269–275)
16. Westphal, P. and G. Christensen. "Lime Stabilization: Effectiveness of Two Process Modifications", J. Wat.Poll. Control Fed., Vol. 55, No. 11, 1983, pp. 1381–1386.
17. Yeager, J. and R. Ward. "Effects of Moisture cvontent on Long-Term Survival and Regrowth of Bacteria and Wastewater Sludge". *App. and Env. Microb.* Vol. 41, No.5, 1981, 1117–1122.
18. Brooks, J., B. Tanner, C. Gerba, and I. Pepper. "The Measurement of Aerosolized Endotoxin From Land Application of Class B Biosolids in Southeast Arizona", *Can. J. Microbiol.* Vol. 52, 2006, pp. 150–156.
19. Castellan, R., S. Olenchock, K. Kinsley, and J. Hankinson. "Inhaled Endotoxin and Decreased Spirometric Values". *New Eng. J. Med.* Vol. 317, No. 10. 1987, pp. 605–610.
20. Laitinen, S. "Importance of Sampling Extraction, and Preservation for the Quantification of Biologically Active Endotoxin", *Env. Med.,* Vol. 6, 1999, pp: 33–38.
21. BioWhittaker. 1996. *Limulas Amebocyte Lysate Assay.* Walkersville, MD.
22. SAS. 2003. *Statistical Analysis Software* Version 9.1. Cary, NC.
23. Carty, C. , U. Gehring, J. Cyrus, W. Bischof, and J. Heinreich. "Seasonal Variability of Endotoxin in Ambient Fine Particulate Matter". *J. Env. Monit.* Vol. 5, No. 6, 2003, pp. 953–958.
24. Milton, D. 1996. *Bacterial Endotoxins: A Review of Health Effects and Potential Impact in the Indoor Environment,* Ind. Air and Human Health, 2cd edition, R. Gammage and B. Bervan, editors, CRC Press Inc., Lewis Publishers, Boca Raton, FL.
25. Simpson,J., R. Niven, C. Pickering, L. Oldham, A. Fletcher, and H. Francis. "Comparative Personal Exposure to Organic Dusts and Endotoxin", *Ann.Occ.Hyg..* Vol. 43, No. 2, 1999, pp. 107–115.
26. ACGIH. 1999. *Bioaerosols: Assessment and Control.* Janet Macher, editor. American Conference of Government Industrial Hygienist. Cincinnati, OH.
27. Epstein, E., N. Wu, C. Youngberg, and G. Croteau. "Controlling Dust and Bioaerosols at a Biosolids Compost Facility". *BioCycle.* Vol. 42, No. 4, 2001, pp. 50–54.
28. NIOSH, 1998. *Criteria for a Recommended Standard: Occupational Exposure to Metal Working Fluids. Publication,* No. 98-102. Cincinnati, OH.

APPENDIX D

Soil Agronomic Results for Land Samples and Fecal Coliform Results for Land Samples

Table D-1. Soil Agronomic Results for Land Samples

SOIL CHARACTERIZATION																			
Sample Date	Sample ID	% Sand	% Silt	% Clay	USDA Textural Class	Bulk Density (gm/cc)	Cation Exchange Capacity (meg/100g)	% Moisture @1/3 bar	% Moisture @15 bar	% Organic Matter	pH (H2O)	% Total N	Total P (ppm)	Olsen Phosphorus (ppm)	Soluble Salts (mmhos/cm)	Base Saturation Data (ppm)				
																K	Ca	Mg	Na	H
8/25/04	PLOT 1 0-5 CM	43.0	28.0	29.0	CLAY LOAM	0.79	12.0	33.0	20.8	8.0	5.9	1.075	1159	45	0.14	150	925	273	26	46
8/25/04	PLOT 1 10-15 CM	55.0	6.0	39.0	SANDY CLAY	1.01	10.8	27.7	17.7	1.9	6.2	2.926	495	8	0.11	33	883	206	23	45
8/25/04	PLOT 1 20-25 CM	29.0	30.0	41.0	CLAY	0.99	10.2	32.7	21.3	1.1	6.8	0.850	330	3	0.10	25	898	190	23	39
8/25/04	PLOT 2 0-5 CM	43.0	32.0	25.0	LOAM	0.87	12.9	31.3	18.6	5.2	6.5	0.574	1079	37	0.26	178	1124	252	20	46
8/25/04	PLOT 2 10-15 CM	35.0	30.0	35.0	CLAY LOAM	1.05	9.9	24.9	14.7	1.8	6.4	0.299	446	4	0.07	57	802	203	22	39
8/25/04	PLOT 2 20-25 CM	29.0	28.0	43.0	CLAY	1.01	9.6	29.8	19.5	1.1	6.9	0.252	301	4	0.12	50	819	197	18	37
8/25/04	PLOT 3 0-5 CM	47.0	26.0	27.0	SANDY CLAY LOAM	0.75	12.3	NA	NA	8.3	5.9	0.729	1209	33	0.14	211	1007	252	24	46
8/25/04	PLOT 3 10-15 CM	33.0	24.0	43.0	CLAY	0.97	9.8	29.7	18.5	2.3	6.7	0.356	424	6	0.20	65	797	227	21	37
8/25/04	PLOT 3 20-25 CM	35.0	20.0	45.0	CLAY	0.90	10.0	38.4	25.0	1.6	6.8	0.363	339	4	0.13	54	773	233	20	40
10/1/04	Biosolid stockpile	5.0	78.6	16.4	SILT LOAM	0.89	22.0	158.4	75.6	NA	7.4	NA	24453	176	4.09	823	2231	627	221	26
9/30/04	P1-A6-0-5 CM	38.8	20.7	41.0	CLAY	NA	14.1	32.3	17.3	6.1	6.5	0.595	1183	NA	0.35	293	1351	334	18	38
9/30/04	P1-A6-10-15 CM	39.0	18.6	42.4	CLAY	NA	10.3	23.2	13.6	2.0	6.4	0.346	493	NA	0.14	48	898	193	12	40
9/30/04	P1-A6-20-25 CM	17.7	23.6	58.7	CLAY	NA	10.5	31.9	22.2	0.8	6.7	0.181	275	NA	0.08	60	917	225	10	38
9/30/04	P1-D0-0-5 CM	30.1	20.2	49.7	CLAY	NA	12.1	31.3	17.6	6.0	5.8	0.554	1313	NA	0.22	173	926	265	12	48
9/30/04	P1-D0-10-15 CM	27.4	27.6	45.0	CLAY	NA	10.0	26.3	16.4	1.6	6.2	0.272	392	NA	0.07	33	821	205	27	40
9/30/04	P1-D0-20-25 CM	20.1	27.8	52.1	CLAY	NA	10.6	32.1	20.3	1.0	6.4	0.141	424	NA	0.07	36	922	225	14	40
9/30/04	P1-G8-0-5 CM	35.1	25.1	39.8	CLAY LOAM	NA	11.7	34.4	17.2	6.6	6.4	0.457	1133	NA	0.29	139	1008	281	18	38
9/30/04	P1-G8-10-15 CM	32.9	30.2	36.9	CLAY LOAM	NA	9.4	23.5	14.3	1.9	6.4	0.326	608	NA	0.07	23	758	195	14	38
9/30/04	P1-G8-20-25 CM	19.2	31.9	48.9	CLAY	NA	9.9	28.6	19.5	0.8	6.7	0.128	396	NA	0.06	21	834	226	13	38
9/30/04	P2-B2-0-5 CM	36.4	22.8	40.8	CLAY	NA	11.2	32.6	17.5	7.0	5.9	0.548	1373	NA	0.20	196	818	256	9	44
9/30/04	P2-B2-10-15 CM	39.0	23.4	37.6	CLAY LOAM	NA	9.0	22.7	13.4	1.9	6.2	0.111	609	NA	0.11	57	697	211	9	36
9/30/04	P2-B2-20-25 CM	24.9	29.6	45.5	CLAY	NA	9.6	28.1	18.0	1.0	6.6	0.084	350	NA	0.10	66	780	227	12	36
9/30/04	P2-J1-0-5 CM	37.1	24.2	38.7	CLAY LOAM	NA	11.3	22.4	14.0	6.2	6.0	0.386	1223	NA	0.15	93	922	258	14	42
9/30/04	P2-J1-10-15 CM	15.9	56.5	27.6	SILTY CLAY LOAM	NA	9.4	30.5	15.2	1.5	6.4	0.155	428	NA	0.09	21	759	203	13	38
9/30/04	P2-J1-20-25 CM	23.2	33.7	43.1	CLAY	NA	9.7	30.4	18.4	0.9	6.8	0.158	231	NA	0.07	20	830	234	13	35
9/30/04	P2-I0-0-5 CM	35.0	25.8	39.2	CLAY LOAM	NA	11.3	31.3	15.4	6.4	5.5	0.363	993	NA	0.14	132	821	236	10	48
9/30/04	P2-I0-10-15 CM	37.3	22.0	40.7	CLAY	NA	8.9	24.6	13.7	1.7	6.6	0.185	404	NA	0.07	27	741	195	11	34
9/30/04	P2-I0-20-25 CM	23.5	25.1	51.4	CLAY	NA	9.7	30.7	19.1	1.1	6.8	0.235	301	NA	0.07	22	845	219	10	35
9/30/04	P3-B8-0-5 CM	36.2	17.2	46.6	CLAY	NA	13.2	34.7	18.5	9.4	6.2	0.353	1183	NA	0.19	152	1196	344	12	39
9/30/04	P3-B8-10-15 CM	37.2	18.5	44.3	CLAY	NA	9.6	24.3	15.9	2.0	6.9	0.450	387	NA	0.13	38	802	268	21	31
9/30/04	P3-B8-20-25 CM	19.2	18.7	62.1	CLAY	NA	9.8	43.1	26.8	0.9	7.1	0.171	303	NA	0.10	27	757	272	11	36
9/30/04	P3-G2-0-5 CM	40.7	14.6	44.7	CLAY	NA	12.5	35.7	18.4	8.2	6.1	0.568	1063	NA	0.29	199	1053	308	21	41
9/30/04	P3-G2-10-15 CM	39.3	16.8	43.9	CLAY	NA	9.1	26.1	15.4	1.9	6.8	0.222	471	NA	0.11	28	758	246	15	31
9/30/04	P3-G2-20-25 CM	20.1	11.0	68.9	CLAY	NA	10.4	41.0	25.5	1.2	6.9	0.104	311	NA	0.12	32	849	294	12	36
9/30/04	P3-J1-0-5 CM	40.6	14.4	45.0	CLAY	NA	13.2	36.7	18.5	7.4	6.2	0.719	1203	NA	0.34	185	1169	408	14	34
9/30/04	P3-J1-10-15 CM	37.5	16.9	45.6	CLAY	NA	9.8	27.3	16.7	1.9	7.1	2.250	418	NA	0.14	32	857	305	14	29
9/30/04	P3-J1-20-25 CM	30.8	14.8	54.4	CLAY	NA	10.2	29.5	18.2	1.5	7.2	0.262	394	NA	0.11	23	901	300	11	31
10/28/04	P1-0-5 CM	42.0	32.0	26.0	LOAM	0.83	11.6	34.4	22.3	7.0	5.7	0.386	1339	NA	0.26	149	915	256	13	45
10/28/04	P1-10-15 CM	36.0	30.0	34.0	CLAY LOAM	1.07	10.2	24.4	15.6	2.1	6.2	0.225	526	NA	0.08	29	832	187	10	43

Table D-1 (continued). Soil Agronomic Results for Land Samples

SOIL CHARACTERIZATION CONT.																			
Sample Date	Sample ID	% Sand	% Silt	% Clay	USDA Textural class	Bulk Density (gm/cc)	Cation Exchange Capacity (meg/100g)	% Moisture @1/3 bar	% Moisture @15 bar	% Organic Matter	pH (H2O)	% Total N	Total P (ppm)	Olsen Phosphorus (ppm)	Soluble Salts (mmhos/cm)	Base Saturation Data (ppm)				
																K	Ca	Mg	Na	H
10/28/04	P1-20-25 CM	30.0	28.0	42.0	CLAY	1.04	10.1	27.7	18.1	1.3	6.4	0.329	313	NA	0.05	25	876	189	11	40
10/28/04	P2-0-5 CM	42.0	26.0	32.0	CLAY LOAM	0.86	12.8	34.1	21.1	7.0	5.8	0.443	1149	NA	0.61	328	985	275	14	46
10/28/04	P2-10-15 CM	36.0	28.0	36.0	CLAY LOAM	1.05	9.4	24.6	14.9	1.7	6.3	0.144	425	NA	0.11	47	740	195	9	39
10/28/04	P2-20-25 CM	32.0	28.0	40.0	CLAY LOAM	1.04	10.0	28.9	18.1	1.2	6.7	0.148	334	NA	0.08	47	852	205	10	38
10/28/04	P3-0-5 CM	36.0	30.0	34.0	CLAY LOAM	0.92	11.8	35.9	20.9	6.4	6	0.309	970	NA	0.19	167	952	255	10	45
10/28/04	P3-10-15 CM	36.0	24.0	40.0	CLAY LOAM	1.05	10.3	26.6	17.6	1.8	6.8	0.114	443	NA	0.10	41	899	259	11	35
10/28/04	P3-20-25 CM	32.0	20.0	48.0	CLAY	0.91	11.9	40.6	29.4	1.4	7.1	0.188	347	NA	0.14	41	994	305	13	42
1/4/2005	P1-0-5 CM	51.0	24.0	25.0	SANDY CLAY LOAM	0.80	12.9	39.8	22.5	8.9	5.7	0.430	1214	49	0.22	172	1012	300	14	48
1/4/05	P1-10-15 CM	41.0	26.0	33.0	CLAY LOAM	1.04	12.2	28.1	17.8	1.8	6.7	0.100	430	8	0.20	34	1123	259	14	42
1/4/05	P1-20-25 CM	45.0	24.0	31.0	SANDY CLAY LOAM	0.99	11.0	32.6	21.6	1.1	6.5	0.070	342	8	0.11	27	948	226	11	42
1/4/05	P2-0-5 CM	49.0	26.0	25.0	SANDY CLAY LOAM	0.87	12.7	29.5	18.8	6.4	5.8	0.370	925	48	0.19	206	1061	290	12	44
1/4/05	P2-10-15 CM	41.0	28.0	31.0	CLAY LOAM	1.07	9.8	24.9	14.6	1.7	6.4	0.100	394	6	0.11	60	816	214	12	38
1/4/05	P2-20-25 CM	39.0	28.0	33.0	CLAY LOAM	1.08	10.0	29.8	18.6	1.1	6.7	0.060	268	6	0.13	48	891	217	11	36
1/4/05	P3-0-5 CM	51.0	18.0	31.0	SANDY CLAY LOAM	0.83	12.9	35.8	21.2	8.6	5.9	0.410	1224	44	0.15	152	1102	302	11	44
1/4/05	P3-10-15 CM	47.0	20.0	33.0	SANDY CLAY LOAM	1.02	10..2	26.4	17.9	1.8	7.2	0.090	383	7	0.12	33	961	284	12	29
1/4/05	P3-20-25 CM	31.0	20.0	49.0	CLAY	0.92	11.7	39.8	26.7	1.3	7.2	0.060	298	5	0.11	41	1090	308	15	35
2/8/05	0Test Soil/ 100Diluent Soil	73.0	8.0	19.0	SANDY LOAM	0.91	8.1	20.3	11.1	3.8	7.6	0.047	51	12	0.23	22	1331	49	36	8
2/8/05	25Test Soil/ 75Diluent Soil	63.0	12.0	25.0	SANDY CLAY LOAM	0.92	13.2	25.1	14.4	4.4	7.6	0.094	292	24	0.23	44	2183	100	35	12
2/8/05	50Test Soil/ 50Diluent Soil	51.0	16.0	33.0	SANDY CLAY LOAM	0.95	16.4	29.1	16.3	5.4	7.5	0.156	531	36	0.30	61	2627	142	26	18
2/8/05	75Test Soil/ 25Diluent Soil	49.0	16.0	35.0	SANDY CLAY LOAM	0.95	20.9	35.2	19.0	5.4	7.4	0.231	870	41	0.33	88	3300	195	23	24
2/8/05	100Test Soil/ 0Diluent Soil	37.0	26.0	37.0	CLAY LOAM	0.96	23.7	37.7	21.1	5.8	7.3	0.289	1238	48	0.47	115	3615	264	13	30

Table D-2. Fecal Coliforms Results for Land Samples

Plot	Date	Fecal Coliforms by Grid Location (MPN/g dry wt)		
		Replicate 1	Replicate 2	Replicate 3
1	8/25/2004	<0.22	<0.22	<0.22
	9/27/2004	<0.23	2.15	<0.22
	9/30/2004	>2.21E03	67.8	2.20E+03
	10/14/2004	>2.27E05	>2.1E05	1.48E+03
	10/28/2004	7.00E+03	6.02E+03	1.23E+04
	12/7/2004	974	1.36E+04	2.38E+03
	1/4/2005	>1.95E04	2.25E+03	1.88E+03
2	8/25/2004	<0.23	<0.22	<0.22
	9/27/2004	0.54	0.55	<0.23
	9/30/2004	>2.24E03	614	>2.15E03
	10/14/2004	1.72E+04	1.43E+04	2.27E+04
	10/28/2004	>2.13E04	>1.96E04	>2.05E04
	12/7/2004	>2.19E04	>2.32E04	1.22E+04
	1/4/2005	2.38E+04	>2.09E04	>2.18E04
3	8/25/2004	<0.23	<0.23	<0.22
	9/27/2004	<0.23	448	<0.22
	9/30/2004	>2.26E03	>2.12E03	>2.24E03
	10/14/2004	4.61E+04	4.43E+04	1.19E+05
	10/28/2004	2.28E+03	1.25E+03	1.21E+04
	12/7/2004	115	239	44
	1/4/2005	2.26E+04	1.95E+03	2.21E+04
Biosolids	10/1/2004	>8.08E06	>8.08E06	>8.08E06