Detection of Aerosolized Endotoxin from a Land Application of Biosolids Site

Brooks, J. P.*, B.D. Tanner, C.P. Gerba, and I.L. Pepper

University of Arizona

Bldg 90 Rm 410

Tucson, Az 85721

* Presenting Author: email jbrooks@arizona.edu

ABSTRACT

Throughout the United States, it has been estimated that approximately 6.5 million tons of biosolids are produced and that 60% of this is land applied. Class B biosolids are land applied in locations typically devoid of communities, but as populations increase and the line between urban and suburban areas begin to blur, biosolids land application is increasingly coming into contact with neighboring communities. Endotoxin (Gram-negative bacteria derived lipopolysaccharide) has been associated with pulmonary ailments, asthma, and fever depending on length of exposure and susceptibility. Aerosol samples were collected from sites in southeastern Arizona from biosolids land application sites. Three SKC Biosamplers were utilized simultaneously, operating at an airflow rate of 12.5 L/min for duration of 10 minutes. Samples were collected at multiple downwind distances either from "loading", "slinging", or "total operation" procedures. All were assayed utilizing the commercially available *Limulus* amebocyte lysate assay. Aerosol samples collected downwind from biosolids loading situations consistently averaged endotoxin concentrations less than half of the maximum recommended endotoxin air concentration, 0.1 $\mu g/m^3$ as suggested by other studies. These levels were similar to endotoxin concentrations during tractor operation on a dry field. At distances greater than 10 m downwind of the point source (land application operations), levels of endotoxin began to decrease, approaching background levels beyond 100 m. Biosolids slinging samples contained aerosol endotoxin concentrations greater than background levels, but were significantly less than loading samples. Overall the amount of endotoxin aerosolized during biosolids operations was below recommended occupational exposure levels for indoor environments, and decreased to background levels beyond 100 m, thus occupational exposure is of greatest concern. When compared to concentrations of aerosolized indicator microorganisms, and subsequently assumed concentrations of pathogens detected at biosolids application sites, endotoxin may be of greater concern to the occupationally exposed.

KEYWORDS
Endotoxin, Aerosol, Biosolids, Pathogen, Bioaerosol

INTRODUCTION

In the United States, greater than 60% of all biosolids produced are land applied, and though this process has occurred for decades, it has recently come under intense scrutiny from communities that neighbor land application sites (National Research Council 2002). Class B biosolids are the treated solid by-product of wastewater treatment that routinely contains pathogenic microorganisms. During a typical land application of "cake" (thickened) biosolids operation, bioaerosols can be aerosolized during biosolids truck unloading, applicator loading, and application operations. In addition bioaerosols can be potentially aerosolized post application, during dry conditions, however recent work in the field has revealed no such phenomenon (data not shown). Biosolids are typically unloaded on site using a "dump" truck, and loaded into a biosolids applicator using a front-end loader. Thickened [MS1]bbiosolids are applied to fields using either a manure spreader or slinger. Biological aerosols (bioaerosols) are biological particles, including pathogenic microorganisms, which have become aerosolized through either human activity such as the land application of biosolids, or through natural activities such as the dispersion of fungal spores. Pathogenic microorganisms and their by-products can all potentially be aerosolized from biosolids during operations. The evaluation of biological aerosols from the land application of biosolids has not been well studied, particularly with regards to endotoxin exposures. Endotoxin is the highly immunogenic by-product of gram-negative bacteria. Common side effects associated with endotoxin aerosol exposures are chest tightness, decreased lung capacity, and fever (Bradley 1979). This study focused on the aerosolization of microorganisms and their by-products associated with the land application of anaerobically digested Class B biosolids.

METHODOLOGY

Phoenix, Az – Site 1

Biosolids application sites were visited throughout the Phoenix, Az metropolitan area. All site operations involved the application of thickened (20%) Class B biosolids. Associated biosolids operations involved loading and slinging operations in which biosolids were loaded into a biosolids application slinger through the use of a front-end loader. Biosolids were then applied through the use of manure slingers in which the biosolids were launched approximately 50 feet into the air.

Mojave, Az – Site 2

"Cake" biosolids were land applied to cotton fields via the use of a Knight Protwin® slinger (Kuhn Knight Inc; Brodhead, WI). Biosolids were launched from the applicator approximately 30 m into the air. This approach provided two different opportunities for sample collection; specifically samples were collected from loading and slinging operations.

Solano, Ca – Site 3

"Cake" biosolids were land applied to grass pasturelands via the use of a modified manure spreader. Through the action of the manure spreader, biosolids were applied from approximately 1 m above the ground and 10 m behind the apparatus. Aerosol samples were collected from loading, spreading, and truck unloading operations.

Bioaerosol Samples - Endotoxin

Bioaerosol samples were collected via the use of six SKC Biosamplers® (SKC-West Inc.; Fullerton, CA) operating at an airflow rate of 12.5 L/min. All biosamplers were sterilized via the use of a steam autoclave prior to field sampling. Following sterilization, a depyrogenation step was included to remove possible endotoxin contaminants. The sterile depyrogenated samplers were placed onto surveying tripods raised to a height so that the intake nozzle simulated the average human breathing height of 1.5 m. Samplers were located perpendicular to the wind vector and placed downwind of thickened biosolids operations, including loading, application, and background operations. In addition endotoxin samples were collected downwind of a wastewater treatment plant aeration basin, and downwind of a tractor operation without biosolids. A total of 3 samplers were used for each sample collection period and were loaded with 20 ml sterile non-pyrogenic water. These were operated for a total of 10 minutes beginning 1 minute prior to exposure of the aerosol plume. Samples were aseptically removed via the use of sterile polystyrene pipettes (Corning; Acton, MA) and were transferred to sterile polystyrene 50 ml centrifuge tubes (VWR; West Chester, PA). All samples were placed on ice until brought back to the laboratory. Samples were then transported back to the laboratory and frozen prior to analysis. Environmental conditions were monitored via the use of a Kessler portable weather monitor during collection of the aerosol samples.

Bioaerosol Samples – Microorganisms

Biological aerosol samples were collected via the use of six SKC Biosamplers® (SKC-West Inc.; Fullerton, CA). Vac-U-Go ® sampling pumps (SKC-West Inc.; Fullerton, CA) were employed to provide a constant air sampling rate of 12.5 l min^{-1}. All samples were collected at a height of 1.5 m, set atop of aluminum tripods (Seco Mfg.; Redding, CA) (ASTM 2004). Samples were collected for a total of 20 min, or approximately 250 l of sampled air. Biosamplers were loaded with 23 ml of 0.1 % peptone buffer amended with antifoam agent B (Sigma-Aldrich; St. Louis, MO). Following sample collection, all were placed on ice and transported overnight for analysis. Prior to analysis, samples were brought back to volume (23 ml) with 0.1 % peptone buffer and vortexed for 1 min. Weather conditions were monitored through the use of a Kestrel portable weather monitor (Nielsen-Kellerman; Boothwyn, PA).

Endotoxin

Aerosolized endotoxin concentrations were determined via the use of a commercially available *Limulus* amebocyte lysate assay. Samples were analyzed in duplicate including multiple dilutions. In addition a control standard endotoxin, spike controls, and appropriate negative controls were included to determine assay efficiency and inhibition. Samples were incubated in 96 well microtiter plates at 37° C for 27 minutes. Following incubation, absorbance readings at 360 nm were collected and sample endotoxin concentrations were determined.

HPC

Aerosolized heterotrophic plate count (HPC) bacteria were assayed in triplicate utilizing R2A media via the spread plate method. An aliquot of the aerosol sample (0.1 ml), including serial dilutions were spread onto R2A media (Becton Dickinson; Sparks, MD) and incubated at 25º C for 7 d. R2A facilitated the enumeration of potentially damaged aerosolized bacteria. Aerosol samples were reported as Colony Forming Units (CFU) m^{-3}.

Coliphage

Aerosolized coliphage able to infect *E. coli* ATCC 15597 was assayed utilizing the double agar overlay technique (Adams 1959). A total of 4 ml from the aerosol sample was assayed using this method. Aerosol samples were reported as Plaque Forming Units (PFU) m^{-3}.

Total Coliform and *Escherichia coli*

Aerosolized total coliform bacteria and *Escherichia coli* were assayed utilizing the commercially available Colilert® enzyme assay (IDEXX; Westbrook, ME) coupled with the Quantitray® Most Probable Number method (American Public Health Association *et al*. 1998). A total of 5 ml of the aerosol sample was assayed utilizing this method. Aerosol samples were reported as Most Probable Number (MPN) m^{-3}.

Clostridium perfringens

Clostridium perfringens was assayed using membrane filtration onto modified mCP media (Acumedia Manufacturers; Baltimore, MD) (Arnon and Payment 1988). All samples were heat shocked at 70° C for 20 min prior to sample analysis. Heat shocking results in enumeration of clostridia spores as vegetative cells are inactivated through the use of heat (Arnon and Payment 1988). A total of 5 ml of the aerosol sample was filtered through a membrane filter (0.45 µm) and aseptically transferred to the media. Petri dishes were then incubated for 1-2 d at 44.5º C in an anaerobically sealed jar (Becton Dickinson Microbiology Systems; Sparks, MD), with anaerobic conditions provided by GasPak Plus (Becton Dickinson Microbiology Systems; Sparks, MD). Aerosol samples were reported as CFU m^{-3}.

RESULTS

Site 1 involved the collection of bioaerosols, which were analyzed for the presence of endotoxin (Fig 1). Samples collected from downwind of biosolids operations involving loading of biosolids were found to contain endotoxin concentrations above 100 EU m^{-3} air and upon occasion approached 1000 EU m^{-3}. On average aerosolized endotoxin concentrations were approximately 344 EU m^{-3}. Samples collected during slinger operation yielded an average concentration of 33.5 EU m^{-3}, while samples collected downwind of the total operation yielded an average concentration of 133.9 EU m^{-3}. To determine the amount of endotoxin contributed by soil aerosolization, samples were collected downwind of tractor operation on a field with no biosolids. Samples averaged 470 EU m^{-3}, which is statistically similar to samples collected during biosolids loading operations. In addition to these, background samples were collected at an offsite location and averaged an aerosol density of approximately 3 EU m^{-3}.

Site 2 involved sample collection from biosolids land application sites involving slinger operation. All samples were analyzed for the presence of culturable microorganisms. Loading samples collected between distances of 2 m and 10 m from site 2 contained elevated levels of indicator bacteria such as total coliforms, *E. coli*, and *C. perfringens* although none were elevated at a statistically significant level. HPC bacteria concentrations were greater than background concentrations, and often times were two $\log_{10}$ greater than background levels. HPC aerosol concentrations involved with loading scenarios were significantly greater than those from slinging samples, which were found to only contain HPC bacteria at concentrations 0.5 $\log_{10}$ greater than background concentrations. No coliphage was detected at this site.

Site 3 involved aerosol collection from operations in which "cake" biosolids were spread via modified manure spreaders. All samples collected were analyzed for the presence of culturable microorganisms. Site 3 HPC concentrations from loading processes were statistically elevated over background, unloading, and spreading concentrations ($P < 0.05$). Total coliforms, *E. coli*, and *C. perfringens* were all detected during loading processes. Total coliform bacteria were detected in all samples collected from loading sites at distances between 2 m and 15 m, although concentrations decreased by two $\log_{10}$ to 10^2 MPN m^{-3} at 15 m ($P < 0.05$). Similar results were obtained from *E. coli* aerosol concentrations downwind of loading situations. *C. perfringens* was detected at low concentrations from loading events and was often barely above detection limits. "Unloading" events yielded *C. perfringens* upon one occasion, but no other indicator microorganisms were detected. Aerosolized HPC bacteria were detected at concentrations similar to background concentrations as no statistical difference was noted between unloading and background aerosol samples. Spreading operations yielded *C. perfringens* on one occasion only, while HPC bacteria were detected at approximately 0.5 $\log_{10}$ greater than background HPC concentrations. These concentrations decreased to levels similar to background concentrations ($P < 0.05$) beyond 28 m. No coliphage was

detected at this site.

DISCUSSION

Overall few aerosolized indicator microorganisms were detected during biosolids operations, while endotoxin was detected more readily. Indicator bacteria were detected infrequently and at low concentrations throughout the study. However, coliform bacteria were detected at the greatest concentration and frequency during loading operations, probably due to the nature of the operation (stationary operation). In addition concentrations of HPC bacteria were detected at greater levels during loading operations, due to the inclusion of soil in the operation. Samples collected during application operations yielded few detectable concentrations of indicator microorganisms, probably due to the nature of the operation (non-stationary operation).

Detectable levels of aerosolized endotoxin were always present during background and biosolids operations. Compared to the amount of indicator microbes aerosolized during these operations, endotoxin is readily present whereas microbial pathogens are assumed to be aerosolized far less frequently, and hence may pose less risk. During loading conditions, dry soil was incorporated into the front-end loader and thus also aerosolized, possibly contributing to overall high levels of aerosolized endotoxin. To avoid this situation, some biosolids application procedures involve the unloading of biosolids onto a metal bin, however this was not the case at any of the sites. Samples collected during conditions in which no biosolids were land applied, but the use of a tractor was employed to aerosolize soil yielded similar aerosolized endotoxin concentrations, thus reinforcing the idea that soil contributes much of the aerosolized endotoxin during biosolids operations. The average concentrations of endotoxin aerosolized during biosolids operations, particularly loading operations, is concentrated enough to cause minor complaints among those populations more highly exposed. However the amount of aerosolized endotoxin contributed just due to biosolids is unknown as soil aerosolization and biosolids operations yielded similar concentrations. In addition, the biological activity associated with aerosolized endotoxin from both soil and biosolids is unknown and warrants further study, as not all endotoxin is biologically active and capable of inducing immune system reactions.

CONCLUSIONS

On average the amount of endotoxin aerosolized during biosolids operations was relatively low when compared to other types of agricultural and industrial sources of aerosolized endotoxin (Rylander *et al* 1983, Donham *et al* 2000). Because so little detection of indicator microorganisms occurred throughout this study, aerosolized endotoxin may be of greater concern with regards to biosolids operations. Only during conditions of biosolids loading, do endotoxin concentrations reach levels above recommended thresholds for some occupational exposures (< 1000 EU m^{-3}) (Rylander *et al* 1983, Donham *et al* 2000). It is important to note that most occupational threshold

levels were established for indoor exposures, where as biosolids operation exposures are limited to outdoor exposures. It is also important to note that not all endotoxin is bioactive and capable of soliciting an immune response. Endotoxin derived from biosolids and soil may not be bioactive, because not all endotoxin is created the same and endotoxin derived from one bacterium may not be as bioactive as endotoxin derived from another. As such, levels above 1000 EU m^{-3} may not be hazardous although more information should be gathered regarding endotoxin derived from biosolids and suggested threshold levels.

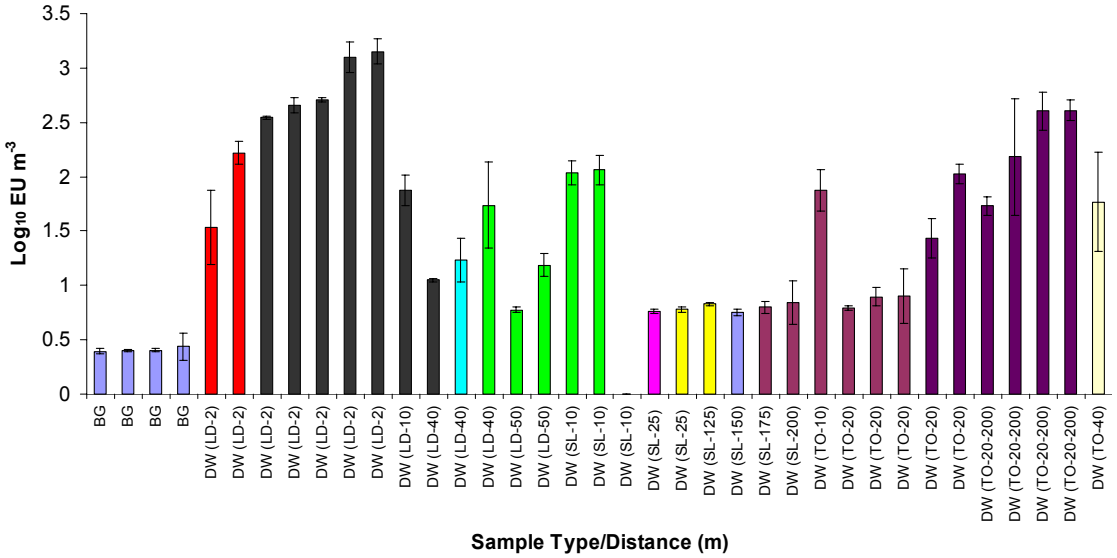

Figure 1. Aerosolized endotoxin concentrations by sample type and distance from source, all bars represent an average of triplicate samples.

* DW – Downwind

† BG – Background, LD – Loading, SL – Slinger, TO – Total Operation

ACKNOWLEDGEMENTS

This project was funded by the National Science Foundation Water Quality Center located at the University of Arizona.

REFERENCES

Adams, M.H. (1959) *Bacteriophages* pp. 27-34. New York, NY: Interscience Publishers, Inc.

American Public Health Association, American Water Works Association, and Water Environment Federation (1998) Enzyme substrate coliform test. In *Standard Methods for the Examination of Water and Wastewater* 20th edition ed. Clesceri, L.S., Greenberg, A.E., and Eaton, A.D. pp. 9-68 – 9-70. Baltimore, MD: United Book Press, Inc.

American Society for Testing and Materials (2004) Standard practice for sampling airborne microorganisms at municipal solid waste processing facilities. In *Annual Book of ASTM Standards Vol. 11.04 Environmental Assessment; Hazardous Substances and Oil Spill Responses; Waste Management* pp. 765-769. W. Conshohocken, PA: ASTM.

Arnon, R. and Payment, P. (1988) A modified mCP medium for enumeration of *Clostridium perfringens* from water samples. *Canadian Journal of Microbiology* **34**, 78-79.

Bradley, S.G. (1979) Cellular and molecular mechanisms of action of bacterial endotoxins. *Annual Review of Microbiology* **33**, 67-94

Donham, K.J., Cumro, D., Reynolds, S.J., and Merchant, J.A. (2000) Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: recommendations for exposure limits. *Journal of Occupational and Environmental Medicine* **42**, 260-269.

National Research Council: Committee on Toxicants and Pathogens in Biosolids Applied to Land (2002) *Biosolids Applied to Land: Advancing Standards and Practices* pp. 1-12. Washington, DC: National Academy Press.

Rylander, R., Lundholm, M., and Clark, C.S. (1983) Exposure to aerosol of microorganisms and toxin during handling of sewage sludge. In *Biological health risk of Sludge Disposal to Land in Cold Climates*. Wallis, P.M., and Lehemann, D.L. (ed). pp. 69-78. Calgary, Alberta: University of Calgary Press.