Effect of Land Application Wastes on Virus Fate

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ABSTRACT

Introduction: Noroviruses cause over 90% of nonbacterial epidemic gastroenteritis worldwide and epidemiological projects in 1999 indicated that noroviruses may account for over 60% of all foodborne disease in the US. Norovirus is shed in the feces of infected humans and animals and can contaminate fresh produce by contamination of the irrigation waters or through contaminated biosolids and manure.

Objective: The objective of this study is to understand factors controlling the survival of viruses in agricultural systems.

Methods: Murine Norovirus Virus 1 (MNV-1, ~ 2*10^5 PFU/ml) was added into two different types of biosolids, pelletized treated (PT) and Alum treated (AT). It was unable to extraction MNV from biosolids with 3% beef extract or Phosphate buffer (Na_3PO_4, pH 9.5) even for sample stored at time 0. Instead, 10 ml phosphate buffer was added to 2 g biosolids, and viral RNA was directly extracted from the solution using Qiagen Viral RNA Extraction Kit (Qiagen, CA). The standard curve of Real-time PCR was generated by serial dilution of biosolids solution, RNA extraction and two-step Real-time PCR with Qiagen SYBR Green Kit (Qiagen, CA). The Most Probable Number Method (MPN) was used to detect the infectivity of MNV in biosolids.

Results: The MNV-1 contaminated biosolids (PT and AT) were incubated at 20 or 4 ºC up to 60 days and analyzed every 10 days. After 60 days incubation, Real-time PCR showed that there was ~5 log reduction of MNV in PT at 20 ºC and ~3 log at 4 ºC; for MNV in AT, there was ~2 log reduction at 20 ºC and 1 log at 4 ºC. The MPN showed that there were ~3 and 4 log PFU/ml MNV still infectious after 60 days in AT at 20 or 4 ºC.

Significance: The survival of MNV-1 is dependent upon biosolid type, treatment, and storage conditions. Overall, MNV is quite stable in biosolids at low temperature and can maintain the ability of infectivity.

INTRODUCTION

The use of animal manures and municipal biosolids on agricultural land remains the most desirable and economically viable option to beneficially recycle these materials. However, animal manures are well-known to contain a variety of microorganisms that are pathogenic to their animal hosts, and biosolids also contain enteric viruses for which humans are the primary source. Application of animal wastes and biosolids to agricultural land can disseminate animal and human pathogens in the natural environment. It has been reported that ground water samples collected from more than 400 public water supply wells located in 35 states contained infectious enteroviruses and rotavirus fragments (Abbaszadegan et al., 2003).

Although viruses are not the only pathogens of concern, they are much smaller in size than other enteric pathogens (e.g., bacteria and protozoa), and can move better through surface. Viruses have been shown to be responsible for approximately 80% of waterborne disease outbreaks for which infectious agents were identifiable and 75% of the estimated cases of foodborne illness. Viruses also tend to survive longer than bacteria and protozoa in the environment and therefore pose greater threats for groundwater and produce contamination. Viruses that are highly mobile and persistent will pose a greater threat in the environment than will viruses that are readily immobilized or have a low potential for survival.

As members of the *Caliciviridae* family, human norovirus (previously known as Norwalk-like viruses) are small (27nm), icosahedral, nonenveloped human enteric viruses that cause acute gastroenteritis. Human noroviruses are the major cause of nonbacterial, epidemic gastroenteritis worldwide and cause significant numbers of endemic cases, as well. It was estimated in 1999 by the CDC that human noroviruses cause 23 million cases of gastroenteritis and 50,000 hospitalization in the United States. Norovirus outbreaks involve people of all ages and often occur in crowded places, such as hospitals, schools, cruise ships and restaurants. Despite the considerable morbidity caused by human noroviruses, no drug vaccine is currently available to treat or prevent human norovirus disease.

Fig 1. Intact core soil columns in the Univ. of Delaware greenhouse.

Norovirus is shed in the feces of infected humans and animals and can contaminate fresh produce by contamination of the irrigation waters or through contaminated biosolids and manure. Norovirus has a high prevalence as an environmental contaminant as shown during the hurricane Katrina aftermath in Louisiana and Houston, where a large number of evacuees contracted gastroenteritis associated with norovirus (CDC, 2005). Since no cell line is yet available for routine cultivation human norovirus, we use Murine norovirus for our study (Wobus et al., 2006). The objective of this study is to understand factors controlling the fate and transport of MNV in agricultural systems.

MATERIALS & METHODS

MNV recovery and extraction. 1ml MNV (~2*10^5 PFU/ml) was added into 2 g pelletized treated biosolids (PT) or Alum treated biosolids (AT) and dried for 10 min. 10 ml phosphate buffer (Na_3PO_4, 0.15 M) or 3% beef extracted was added to MNV contaminated biosolids and vortex for 30 min for virus recovery. The sample was then centrifuged at 18,000xg for 30 min for virus extraction. However, it was unable to extract the MNV from PT or AT with either buffer.

RNA extraction. Instead, 10 ml phosphate buffer was added to 10 g MNV contaminated biosolids and RNA was directly extracted from biosolids solution using Qiagen Viral RNA Extraction Kit (Qiagen, CA). Real-time PCR was constructed by serial dilution of biosolids, RNA extraction and two-step PCR with Qiagen SYBR Green Kit (Qiagen, CA). The titer of initial MNV stock was determined by plaque assay.

Virus infectivity study. Viral infectivity was determined by Most Probable Number (MPN) method. The RAW cells were incubated in 24 well plates. The biosolids solution was serial diluted and added into each well of RAW cells in triplicate. After 2 days incubation, the total RNA of cells were extracted from cell with Trizol Reagent (Invitrogen, California). RT-PCR was conducted using MNV primer and the quantity of infectious MNV was determined by MPN-table.

Survival study. 1ml MNV was added to 2g PT or AT biosolids, stored at 20 or 4°C for 60 days and analyzed every 10 days. The RNA extraction and Real-time PCR was used to detect quantity of viral particle left in biosolids. The infectivity was determined by MPN method.

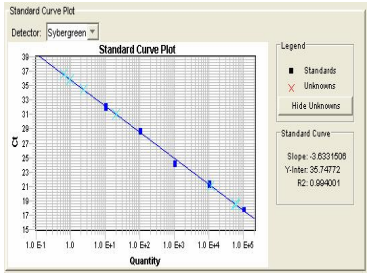

Fig. 2 Standard curve of MNV in AT biosolid

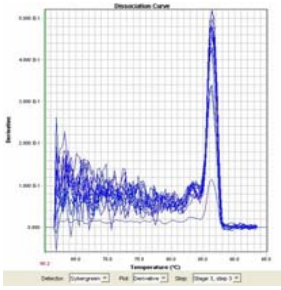

Fig.3 Dissociation curve of MNV in biosolids (serial diluted for standard curve at day 0).

Virus leaching from soil columns amended with manures/biosolids.

Soil column preparation. Polyvinyl chloride (PVC) soil columns were collected to a depth of 0.5 m or 1.0 m using a tractor mounted hydraulic column collector. The bottom 2-cm of soil was removed from the PVC pipe and replaced with a mixture of sand and gravel to create a free-draining situation. The columns was dry for several weeks to induce shrinkage, the silicone sealer was injected into the bottom of the void between the soil column and the pipe wall. To adjust for differences in antecedent moisture content in the soils, all columns were slowly wet to surface saturation and using an automated drip irrigation system.

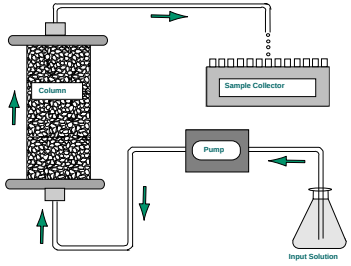

Fig. 4 Setup of virus leaching from soil column

MNV amended in biosolids leaching from soil column. Selected manures and biosolids will be inoculated with MNV (~2*105 PFU/ml), and applied either on the soil surface or incorporated to a depth of 10 cm. Control columns with no manure/biosolids or uninoculated manure/biosolids will also be used. Simulated rainfall will be added on weekly basis for 2 months. All leachate will be collected and analyzed for pH, total OM, total nutrients and total viral concentration.

RESULTS

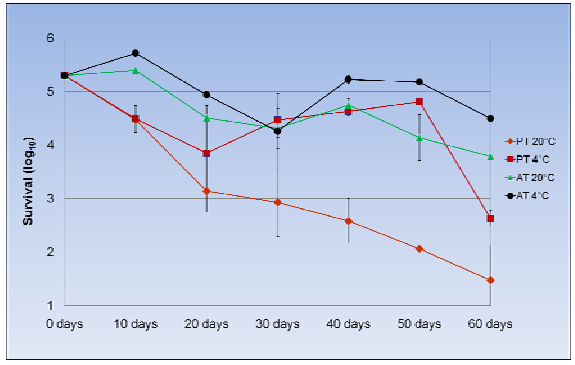

Fig 5. Real-time PCR results of sixty days study of survival of MNV in biosolids. PT: pelletized treated; AT: Alum treated biosolids

Fig 6. Alum treated biosolids (left) and Pelletized biosolids (right)

Future Research

Table 1. Biosolids and manures used for future virus survival study.

Type	Character
Raw poultry litter	One year storage in the field stockpiles
Digested biosolids 1	Digested and lime-stabilized
Digested biosolids 2	Digested and treated with Fe salts
Swine manure	Liquid, from storage lagoon

Four more biosolids (listed in Table 1) will be used for MNV survival study. Also, the fate and transport of two more pathogenic viruses will be assessed (Hepatitis A and Adenovirus 41) within the six soil-amendments. The effect of different properties and management of biosolids and manures on the survival of viruses will be evaluated. Also, the fate of different types of pathogenic viruses will be compared.

Virus fate and transport in small laboratory columns will be conducted. The main objective of the small-scale experiments is to elucidate the mechanisms of virus retention and transport during leaching under saturated and unsaturated flow conditions.

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