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Comparative survival of viruses during thermophilic and mesophilic anaerobic digestion

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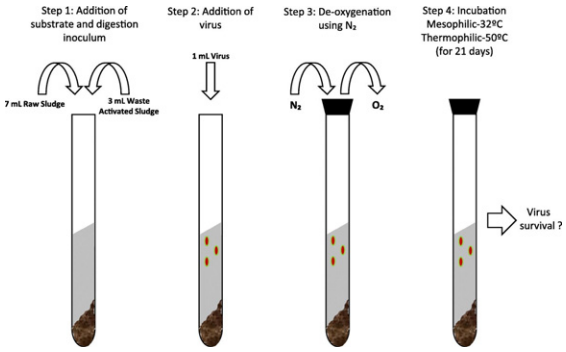
HIGHLIGHTS

- Microscale technology utilized to demonstrate inactivation of viruses during anaerobic digestion.
- Novel data provided on viral inactivation of both animal viruses and bacteriophages during anaerobic digestion.
- Data on mesophilic and thermophilic digestion provided.
- Ø6 shown to be an effective surrogate for Ebola in survival studies.
- MS2 shown to be an inappropriate surrogate for Ebola in survival studies.

GRAPHICAL ABSTRACT



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ABSTRACT

Micro-scale technology was used to obtain survival data for three animal viruses and two bacteriophages during anaerobic digestion. The data for adenoviruses, MS2 and Φ6 provide the first published reports for survival of these viruses during mesophilic anaerobic digestion. Data were also obtained for thermophilic digestion, which showed greater inactivation of viruses at the higher temperature. Data on the survival of Φ6 are of particular interest since it is a lipid-based virus which has been suggested as a surrogate for the Ebola virus. In contrast, MS2 was found to be an inappropriate surrogate for Ebola.

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1. Introduction

During traditional wastewater treatment, anaerobic digestion facilitates the degradation and reduction of organic matter within the waste stream (Gavala et al., 2003). The most widely used type of anaerobic digestion by wastewater treatment plants occurs under mesophilic conditions (30 °C to 40 °C) due to the relatively low-energy usage of this process. However, the U.S. Environmental Protection Agency has also approved the practice of thermophilic digestion (50 °C to 60 °C) for the treatment of sludge to produce Class A biosolids (EPA, 1993). Although more energy-intensive, thermophilic digestion does result in greater inactivation levels of pathogenic microorganisms (Nayono, 2010; Stillwell et al., 2010).

Data detailing the survival of animal viruses and bacteriophages during anaerobic digestion are lacking. Specifically, knowledge gaps exist

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for adenoviruses, MS2 and Φ6 at mesophilic temperatures, and for most viruses at thermophilic temperatures. The survival of human pathogenic viruses is of particular interest due to the possible impact on public health when reclaimed waters or Class B biosolids are reused even for crop irrigation or land application. The potential exposure of wastewater plant personnel to infectious viruses during sewage treatment is also of concern. Finally, the recent appearance of the Ebola virus (EBV) in the United States has led to an increased interest in the survival characteristics of this emerging virus during the process of wastewater treatment. EBV can be excreted in high concentrations in bodily fluids including urine, feces, blood and vomit (up to 10^8/mL) (Bibby et al., 2015). A study evaluating survival of EBV in sterile wastewater showed that the concentration of infectious particles was greatly reduced within the first 24 h (Bibby et al., 2015). However, this laboratory study suffered from two limitations, self-identified by the authors. First, the tested wastewater was more dilute than typical wastewaters, which may have affected the inactivation of the virus. Second, prior to utilization the wastewater was frozen to minimize compositional changes, and disinfected (γ-irradiation prior to utilization to limit microbial activity and false positive cell cultures. However, minimizing microbial activity may have affected the rates of viral inactivation. In addition, the effects of other environmental characteristics, particularly temperature, on virus inactivation were not evaluated. That being said, the study did utilize a true Ebola virus, and provides important additional data to a very limited database on Ebola survival in wastewater. Virus inactivation or adsorption to suspended solids present in the water are two possible reasons for the reduction of infectious viruses (Bibby et al., 2015). Recently, bacteriophage Φ6 bacteriophage has been proposed as a potential surrogate for EBV because both are RNA viruses and both contain a lipid-based envelope (Bibby et al., 2014). Casanova and Weaver (2015) studied the survival of Φ6 in wastewater at 30 °C. In this study Φ6 was spiked into sewage (primary influent having undergone no treatment) obtained from a 40 mgd wastewater reclamation facility. Samples of influent were pasturized at 70 °C for 3 h to suppress microbes that would interfere with the virus assay. Following the spiking of Φ6, viral titers were measured every 24 h. Data show that the virus was inactivated by 5-$\log_{10}$ within 48 h, and >7-$\log_{10}$ within 72 h.

Although anaerobic digestion was not initially designed to remove pathogens, it may effectively reduce the levels of a wide range of pathogens including viruses (EPA, 1993). Unfortunately, little is known about the survival of emerging viruses such as Ebola by mesophilic and thermophilic digestion processes. The goal of this study was to investigate the inactivation of several classes of viruses and bacteriophages during a simulated anaerobic digestion process. There were two specific aims of the study: i) to quantify the removal of a lipid containing virus, murine norovirus, adenovirus and coliphage, during anaerobic digestion of sewage sludge; and ii) evaluate the potential survival of the Ebola virus during anaerobic digestion through the use of surrogates with similar characteristics to the Ebola virus. For this purpose, a novel micro-scale technology was utilized under both mesophilic and thermophilic temperature conditions. Overall, three animal viruses (poliovirus 1, murine norovirus, and adenovirus 4) and two bacteriophages (MS2 and Φ6) were evaluated as potential surrogates for the Ebola virus. These surrogates were chosen based on similarity to Ebola with respect to viral order, lipid content, and/or nucleic acid content.

2. Methods

2.1. Preparation of viruses and permissive mammalian cell cultures

The cell lines used to propagate and assay for the mammalian viruses of interest in the study were Primary Liver Carcinoma (PLC, ATCC No. CRL-8024), murine macrophages (RAW 264.7, ATCC No. TIB-71), and Buffalo Green Monkey kidney cells (BGMs, kindly provided by D. Dahling at the United States Environmental Protection Agency, Cincinnati, OH). The PLC cell line is permissive to adenovirus 4 (Ad4), and was cultivated using modified Eagle's medium (MEM; MediaTech Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) and glucose (Corning, Corning, NY, USA). RAW cells were maintained and assayed in preparation for work with murine norovirus (MNV) using Dulbecco's modified Eagle medium (DMEM; MediaTech Inc., Manassas, VA, USA) amended to obtain a final concentration of 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA). BGM cells were grown using modified Eagle's medium (MEM; MediaTech Inc., Manassas, VA, USA) with 5% calf serum (CS; Hyclone Laboratories, Logan, UT, USA) for the study of poliovirus type 1 (strain LSc-2ab) (PV1). All cell lines were grown to a minimum of 80% confluency for up to seven days at 37 °C in a 5% CO_2 atmosphere incubator prior to sub-culturing and the preparation of 24-well plates. AD4, PV1, and MNV were propagated and purified using methods previously described by Soto-Beltran et al. (2013). All viruses were stored at −80 °C until use.

2.2. Bacteriophage propagation

MS2 (ATCC No. 15597-B1) was propagated and maintained as previously described by Sassi et al. (2015) using host cell lawns of *Escherichia coli* (ATCC No. 15597). The bacterial-host cell system for Φ6 and *Pseudomonas syringae* (ATCC No. 10205) was generously provided by Dr. Krista R. Wigginton at the University of Michigan in Ann Arbor, MI. In order to propagate bacteriophage Φ6, a single colony of *P. syringae* was inoculated into 10 mL of sterile LB broth (BD, Franklin Lakes, NJ, USA), and incubated for 18 h at 26 °C with agitation (120 rpm). The double layer agar overlay technique was then employed by combining 0.2 mL of the *P. syringae* culture with 0.1 mL of bacteriophage Φ6 containing ~10^6 plaque-forming units (PFU) in a LB top agar tube. The tube was then gently poured over a tryptic soy agar plate (TSA; BD, Franklin Lakes, NJ, USA). Upon solidification, the plates were inverted and incubated at 26 °C for 24 h. For plates demonstrating visible plaques of Φ6 on the *P. syringae* host lawn, 6 mL of 0.01 M phosphate-buffered saline (PBS) was pipetted over the entire lawn, followed by incubation at room temperature for 2 h with gentle agitation every 30 min. A sterile cell scraper (Corning®, Corning, NY, USA) was then gently used to further mechanically detach the viruses from agarose medium. The harvested extracts from each plate were combined in sterile conical tube, and centrifuged to pellet debris (1500 × *g*, 15 min). The supernatant containing the virus was then passed through a 0.22 μm pore-sized filter for purification.

2.3. Anaerobic digestion

To simulate the anaerobic digestion process, mini-digesters were constructed using a technology previously utilized in our laboratory to evaluate the influence of this process on the survival of infectious prions (Miles et al., 2011). Briefly, 7 mL of primary sludge (Total Suspended Solids (TSS): 68 mg/L) and 3 mL of waste activated sludge (WAS, TSS: 27,500 mg/L) obtained from the Tres Rios Water Reclamation Facility in Tucson, Arizona were combined in sterile, 16 × 150 mm anaerobic glass tubes (#2048-18150, Bellco Glass, Vineland, NJ, USA). One-milliliter of virus was added to each tube, which was then sealed with a 20 mm septum stopper followed by a 20 mm aluminum seal (#2048-11800; #2048-11020, Bellco Glass, Vineyard, NJ, USA). The tubes were flushed with N_2 gas for 3 min using two sterile 18-gauge syringe needles (BD, Franklin Lakes, NJ, USA) to create an anaerobic environment, with one needle serving solely as an outlet for the oxygenated air being flushed from the tube.

After preparation, the tubes were incubated anaerobically using an anaerobic jar with catalyst packs (GasPak Pouch, BD, Franklin Lakes, NJ, USA) under conditions replicative of either mesophilic digestion (32 ± 3 °C for 21 days ± 8 h), or thermophilic digestion (55 ± 3 °C for 5 days ± 8 h). All digestion processes took place with constant