




# Ammonia as an *In Situ* Sanitizer: Influence of Virus Genome Type on Inactivation

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## ABSTRACT

Treatment of human excreta and animal manure (HEAM) is key in controlling the spread of persistent enteric pathogens, such as viruses. The extent of virus inactivation during HEAM storage and treatment appears to vary with virus genome type, although the reasons for this variability are not clear. Here, we investigated the inactivation of viruses of different genome types under conditions representative of HEAM storage or mesophilic digestion. The goals were to characterize the influence of HEAM solution conditions on inactivation and to determine the potential mechanisms involved. Specifically, eight viruses representing the four viral genome types (single-stranded RNA [ssRNA], double-stranded RNA [dsRNA], single-stranded DNA [ssDNA], and double-stranded DNA [dsDNA]) were exposed to synthetic solutions with well-controlled temperature (20 to 35°C), pH (8 to 9), and ammonia (NH<sub>3</sub>) concentrations (0 to 40 mmol liter<sup>-1</sup>). DNA and dsRNA viruses were considerably more resistant than ssRNA viruses, resulting in up to 1,000-fold-longer treatment times to reach a 4-log inactivation. The apparently slower inactivation of DNA viruses was rationalized by the higher stability of DNA than that of ssRNA in HEAM. Pushing the system toward harsher pH (>9) and temperature (>35°C) conditions, such as those encountered in thermophilic digestion and alkaline treatments, led to more consistent inactivation kinetics among ssRNA and other viruses. This suggests that the dependence of inactivation on genome type disappeared in favor of protein-mediated inactivation mechanisms common to all viruses. Finally, we recommend the use of MS2 as a conservative indicator to assess the inactivation of ssRNA viruses and the stable ΦX174 or dsDNA phages as indicators for persistent viruses.

## IMPORTANCE

Viruses are among the most environmentally persistent pathogens. They can be present in high concentrations in human excreta and animal manure (HEAM). Therefore, appropriate treatment of HEAM is important prior to its reuse or discharge into the environment. Here, we investigated the factors that determine the persistence of viruses in HEAM, and we determined the main mechanisms that lead to their inactivation. Unlike other organisms, viruses can have four different genome types (double- or single-stranded RNA or DNA), and the viruses studied herein represent all four types. Genome type appeared to be the major determinant for persistence. Single-stranded RNA viruses are the most labile, because this genome type is susceptible to degradation in HEAM. In contrast, the other genome types are more stable; therefore, inactivation is slower and mainly driven by the degradation of viral proteins. Overall, this study allows us to better understand the behavior of viruses in HEAM.

Human excreta and animal manure (HEAM) may pose a threat to people because they are a major reservoir for many fecally-orally transmissible pathogenic organisms (1). In addition, untreated HEAM may release chemical contaminants into the environment (e.g., macronutrients and microorganic pollutants or heavy metals). While HEAM thus represents a source of pollution, it simultaneously is a valuable product containing nutrients, e.g., nitrogen, phosphorus, and potassium, which are essential for plant growth (2, 3), and water for irrigation (4). In order to safely harvest these valuable components, adequate waste treatment is a necessity to prevent the spread of diseases and the contamination of the environment.

Many fecally-orally transmissible pathogens, such as viruses, protozoa, and helminthes, are parasitic organisms that cannot reproduce or grow outside their host. Thus, from the time of excretion from the host, the infective pathogen concentration generally declines with time (5). HEAM treatment and stabilization processes may accelerate this decline by creating harsh environmental conditions that promote pathogen inactivation. Effective treatment of HEAM can generate safe soil conditioner or fertilizer products for land application (6). A wide array of processes are

available for sanitation (6, 7), ranging from storage at ambient temperature to chemical treatment with sanitizing substances (8, 9), to aerobic or anaerobic digestion, composting, and alkaline and heat treatment. For most of these processes, the temperature, pH, water content, and the exposure to sanitizing substances, such as ammonia, govern the extent of pathogen inactivation (1, 10). Yet, the individual and synergistic contributions of these parameters to pathogen inactivation remain to be systematically characterized.

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TABLE 1 Typical temperature, pH, and ammonia conditions in human/animal waste during storage and mesophilic anaerobic digestion

Type of waste by treatment type	Range					Reference(s)
	NH <sub>4</sub> <sup>+</sup> /NH <sub>3</sub> (mmol liter <sup>-1</sup> )	Temp (°C)	pH	{NH <sub>3(aq)</sub> } (mmol liter <sup>-1</sup> ) <sup>a</sup>		
<b>Storage</b>						
Urine	5–475	4–35	8.2–9.8	2–246		25, 87, 104
Feces (raw)	80–247	20–34	6.8–8.3	0.2–32		27, 105
Feces (+ additives) <sup>b</sup>	60–862	20–34	7.5–12.8	1–340		
Feces + urine	104–1,098	10–28	8.8–9.2	9–439		106
<b>Anaerobic digestion<sup>c</sup></b>						
Animal manure	35–450	25–38	7.0–8.1	0–37		107–110
Sewage sludge	42–45	37	7.5–8.0	1–5		34
Activated sludge	149–191	37	7.7–7.9	7–13		111

<sup>a</sup> Ammonia activity (evaluated with PHREEQC, as described in Materials and Methods).

<sup>b</sup> Urea, wood ash, or oyster shell.

<sup>c</sup> Under mesophilic conditions.

Among possible sanitizing compounds, total ammonia (NH<sub>4</sub><sup>+</sup>/NH<sub>3</sub>) is of particular interest because it may be naturally present at substantial levels in stored urine, stored fecal sludge, and anaerobically digested sludge (Table 1). Total ammonia is produced by urea and protein hydrolysis during the storage and digestion of HEAM (11, 12). In its neutral dissolved form, aqueous ammonia (NH<sub>3(aq)</sub>) is a major nitrogen source for bacteria, eukaryotic microbes, fungi, and plants. However, NH<sub>3(aq)</sub> may become harmful at elevated concentrations (13); it was found to have biocidal activity against most pathogenic microorganisms (14–18), and it was shown to be the main substance responsible for virus die-off in sludge (19). NH<sub>3(aq)</sub> can thus be considered an *in situ* sanitizer naturally present in HEAM.

The mechanisms involved in the toxicity of NH<sub>3(aq)</sub> toward eukaryotic and prokaryotic cells are not fully understood. Several hypotheses have been put forward to explain its biocidal effect, namely, intracellular pH change, disturbance of the electrochemical gradient across the cell membrane, and inhibition of enzymatic reactions (20–23). Unlike other pathogenic microorganisms, viruses do not have a cell metabolism of their own but use the host machinery to reproduce (24). Therefore, the biocidal processes involving NH<sub>3(aq)</sub> relevant to cells do not apply to viruses. Instead, the virucidal activity of NH<sub>3(aq)</sub> likely involves modification or damage of the virus components (i.e., protein, envelope, or nucleic acid). However, while numerous studies have investigated the fate of viruses in complex matrices containing NH<sub>3(aq)</sub> (8, 17, 19, 25–37), the virucidal mechanism of action of NH<sub>3(aq)</sub> has received little scrutiny to date (38, 39).

Unlike other living organisms, viruses carry their genetic information in different forms, specifically as single-stranded (ss) or double-stranded (ds) RNA or DNA (24). Several studies have observed differences in inactivation behavior among viruses with different genome types (19, 25, 26, 30, 33–36). This suggests that the genome may be an important target of NH<sub>3(aq)</sub>, and that the different genome types differ in their NH<sub>3(aq)</sub> susceptibilities. Other factors besides genome type, such as genome geometry or packaging, capsid properties, or the presence of an envelope, may additionally influence virus stability in the presence of NH<sub>3(aq)</sub>, although the extent to which they influence NH<sub>3</sub>-mediated inactivation is not clear. Given that viruses are generally permeable to water and to salt ions (40, 41), however, none of these structural features should have a greatly protective effect against the action of NH<sub>3(aq)</sub> on the viral genomes.

Here, we exposed a suite of viruses to NH<sub>3(aq)</sub> in controlled laboratory solutions in order to characterize how the genome type influences the kinetics of NH<sub>3(aq)</sub>-mediated inactivation. We focused on naked viruses, which represent the majority of enteric viruses of interest. Furthermore, we put our observations in the context of literature data to elucidate the potential mechanisms of inactivation. Our working hypothesis is that the viral genome type is the major determinant for susceptibility to NH<sub>3(aq)</sub>, and that the influence of other virus-specific parameters, such as genome sequence or protein structure, is minor in comparison. A total of eight viruses representing all genome types were studied: bacteriophages MS2 and GA and human echovirus and coxsackievirus (single-stranded RNA [ssRNA]), mammalian reovirus (double-stranded RNA [dsRNA]), bacteriophage ΦX174 (single-stranded DNA [ssDNA]), and bacteriophage T4 and human adenovirus (double-stranded DNA [dsDNA]). The ultimate goal of this study was to advance our understanding of the dominant mechanisms involved in virus inactivation over a range of environmental conditions relevant to HEAM storage or processing (Table 1).

## MATERIALS AND METHODS

**Definition of virus.** According to Bãndea (42), infective viral particles outside their host should be referred to as virions. Correspondingly, in this study, we were interested in the effect of HEAM treatment and storage on virions. However, viral solutions always contain both virions and non-infective viral particles, and the processes addressed herein act on both. As such, we use the less-specific word “virus” throughout the manuscript, even if it is the virions that are of the most interest.

**Viruses and cells.** Human adenovirus (HAdV) type 2 was kindly provided by Rosina Gironès (University of Barcelona, Spain). Echovirus (EV) type 11 (ATCC VR-41), coxsackievirus (CV) B5 Faulkner (ATCC VR-185), and mammalian reovirus (ReoV) type 1 (ATCC VR-230) were purchased from LGC Standards (Molsheim, France). HAdV, EV/CV, and ReoV were propagated on A549 human lung carcinoma epithelial cells, buffalo green monkey kidney (BGMK) cells, and L929 mouse fibroblasts, respectively. A549 and L929 cells were kindly provided by the Lausanne University Hospital (Switzerland) and BGMK cells by the University of Barcelona. A549 cells were cultivated in high-glucose pyruvate Dulbecco's modified Eagle's medium (DMEM; Invitrogen), and L929 and BGMK cells were cultivated in minimum essential medium (MEM; Invitrogen). Both media were supplemented with penicillin (20 U ml<sup>-1</sup>), streptomycin (20 μg ml<sup>-1</sup>) (Invitrogen), and 2 or 10% fetal bovine serum (FBS; Invitrogen), and cells were incubated at 37°C in 5% CO<sub>2</sub> and 95% humidity. Viruses were propagated by spiking 10 μl of HAdV or CV (10<sup>10</sup> to 10<sup>11</sup> most probable number of cytopathogenic units [MPNCU] ml<sup>-1</sup>) or 100

$\mu\text{l}$  of EV or ReoV ( $10^7$  to  $10^8$  MPNCU  $\text{ml}^{-1}$ ) into 160- $\text{cm}^2$  flasks (TPP Techno Plastic Products, Trasadingen, Switzerland) containing 95% confluent cells. The flasks were incubated until cytopathic effects were apparent. The viruses were then purified as described by Bosshard et al. (43). From each flask, 1 ml of sample containing  $10^{10}$  to  $10^{11}$  MPNCU  $\text{ml}^{-1}$  of HAdV,  $10^9$  to  $10^{10}$  MPNCU  $\text{ml}^{-1}$  of CV,  $10^7$  to  $10^8$  MPNCU  $\text{ml}^{-1}$  of EV, or  $10^7$  to  $10^8$  MPNCU  $\text{ml}^{-1}$  of ReoV was collected and stored at  $4^\circ\text{C}$  as virus stocks for the experiments. New stocks were produced before each set of experiments. Virus titers were determined by most probable number analysis from  $5 \times 100 \mu\text{l}$  of samples on 96-well plates (Greiner Bio-One, Frickenhausen, Germany), as described by Bosshard et al. (43). Briefly, the DMEM/MEM containing 10% FBS on a 95% confluent cell monolayer was replaced by  $100 \mu\text{l}$  of virus solution and augmented with  $200 \mu\text{l}$  of DMEM/MEM containing 2% FBS. Cytopathogenic units could be seen after incubation times of 14, 4 to 7, and 10 days for HAdV, EV/CV, and ReoV, respectively. The detection limit for all viruses was  $10^2$  to  $10^3$  MPNCU  $\text{ml}^{-1}$ .

**Phages and bacteria.** Coliphages MS2 (DSMZ 13767) and  $\Phi\text{X174}$  (DSMZ 4497) and their host *Escherichia coli* (DSMZ 5695 and DSMZ 13127) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Coliphage GA was kindly provided by Joan Jofre (University of Barcelona) and was cultured in the same *E. coli* host as MS2. Coliphage T4 and its *E. coli* B1 host were kindly provided by Petr Leiman (École Polytechnique Fédérale de Lausanne, Switzerland). All phages were propagated and purified as described previously (44), except that *E. coli* B1 was grown in the absence of antibiotics. Stock solutions were kept in virus dilution buffer (VDB) ( $5 \text{ mmol liter}^{-1} \text{NaH}_2\text{PO}_4$ ,  $10 \text{ mmol liter}^{-1} \text{NaCl}$  [pH 7.5]) and were stored at  $4^\circ\text{C}$ . The same stocks were used for all the experiments. Infectivity was assessed using the double-agar-layer method and was expressed as the PFU per milliliter (45). The detection limit for all phages was  $300 \text{ PFU ml}^{-1}$ .

**Chemicals.** Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ; Fluka), sodium bicarbonate ( $\text{NaHCO}_3$ ; Acros), ammonium chloride ( $\text{NH}_4\text{Cl}$ ; Acros), potassium chloride (KCl; Acros), sodium chloride (NaCl; Acros), and sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ; Acros) were used to make the experimental solutions described below.

**Experimental solutions.** Virus inactivation was assessed over a range of temperatures, pH values, and  $\text{NH}_3(\text{aq})$  activities ( $\{\text{NH}_3(\text{aq})\}$ ). Temperature, pH, and  $\{\text{NH}_3(\text{aq})\}$  were chosen within an environmentally relevant range, as detailed in Table 1. pH 8 and  $20^\circ\text{C}$  (pH 8/ $20^\circ\text{C}$ ) represents the baseline conditions from which pH and temperature effects were assessed. For all pH/temperature ( $T$ ) conditions, inactivation was quantified at approximate  $\{\text{NH}_3(\text{aq})\}$  of 0, 20, and  $40 \text{ mmol liter}^{-1}$ . Phosphate carbonate buffer (PCa) was used as the  $\text{NH}_3(\text{aq})$ -free control, whereas ammonium carbonate buffer (AmCa) was used to generate solutions containing  $\text{NH}_3(\text{aq})$ . The specific composition of the buffers to attain a given  $\text{NH}_3(\text{aq})$  activity was determined with PHREEQC (version 2.18.00) (46) and a database using the Pitzer approach for calculating the ion activity, as described by Decrey et al. (38). The exact buffer composition and resulting  $\{\text{NH}_3(\text{aq})\}$  for each experiment can be found in Table S1 in the supplemental material.

Under baseline conditions (pH 8/ $20^\circ\text{C}$ ), the AmCa solutions with  $\{\text{NH}_3(\text{aq})\}$  of  $\approx 40 \text{ mmol liter}^{-1}$  exhibited a high ionic strength compared to the PCa solution. Therefore, control experiments were conducted to distinguish between the effects of ionic strength and  $\text{NH}_3(\text{aq})$ . These control experiments were conducted under baseline conditions in high-ionic-strength PCa (PCaH), where the ionic strength was increased by adding sodium chloride. The ionic strength was assessed by measurement of the electrical conductivity (EC) with a Cond315i conductivity meter and a TetraCon 325 probe (WTW, Weilheim, Germany) (see Table S1 in the supplemental material).

For phages MS2,  $\Phi\text{X174}$ , and T4, additional experiments were conducted at pH 10 and  $11/20^\circ\text{C}$  in carbonate buffer, pH 11.5 and  $12/20^\circ\text{C}$  in phosphate buffer, and at pH  $8/50^\circ\text{C}$  and  $60^\circ\text{C}$  in PCa, to assess the influence of extreme pH and temperature, respectively. Potassium carbonate so-

lutions (KCa) with  $\text{K}^+$  activity similar to  $\text{NH}_4^+$  in AmCa at pH 9 and  $20^\circ\text{C}$  were used to assess the effect of monovalent cations on EV inactivation.

**Experimental setup.** For MS2, GA,  $\Phi\text{X174}$ , T4, and HAdV, 1 ml of a virus solution containing  $10^7$  to  $10^{10}$  PFU or MPNCU  $\text{ml}^{-1}$  in VDB was added to airtight 116-ml glass serum flasks (Infochroma, Zug, Switzerland) containing 114 ml of experimental solution. For the lower-titer ReoV, EV, and CV, 1 ml of virus stock ( $10^6$  to  $10^{10}$  MPNCU  $\text{ml}^{-1}$ ) was added to airtight 16-ml glass serum flasks containing 14 ml of experimental buffer. After mixing, a 1-ml sample was taken from each flask with a sterile syringe and filtered through a  $0.22\text{-}\mu\text{m}$ -pore-size filter (Millipore). The filtered samples were directly diluted in medium containing 2% FBS (HAdV, ReoV, EV, and CV) or VDB (MS2, GA,  $\Phi\text{X174}$ , and T4) and were stored at  $4^\circ\text{C}$  for no more than 6 h prior to enumeration. Each pH,  $T$ , and  $\{\text{NH}_3(\text{aq})\}$  condition was tested in duplicate flasks for all organisms. Phage titers were determined in triplicate from the same flasks, whereas viruses were enumerated once per flask. At the end of each experiments, the pH was measured at experimental temperature, and the  $\text{NH}_4^+/\text{NH}_3$  concentration was determined by ion chromatography (ICS-3000A, IonPac CS16 column) with electrical conductivity detection (Dionex, Switzerland). From these measurements,  $\{\text{NH}_3(\text{aq})\}$  was calculated as described by Decrey et al. (38). In order to stop the fast inactivation at high pH, samples were mixed with HCl prior to dilution in VDB as follows: for pH 11.5,  $940 \mu\text{l}$  of sample was amended with  $60 \mu\text{l}$  of HCl (0.1 N), and for pH 12.0,  $400 \mu\text{l}$  of sample was mixed with  $600 \mu\text{l}$  HCl (0.01 N).

**Data analysis.** Inactivation kinetics were determined by least-square fit of the data to a first-order model according to the following equation:

$$\ln \frac{C}{C_0} = -k_{\text{obs}} t$$

where  $C_0$  and  $C$  (in PFU or MPNCU per milliliter) are the virus concentrations at time zero and time  $t$ , respectively, and  $k_{\text{obs}}$  is the first-order inactivation rate constant ( $\text{day}^{-1}$ ). The data of all replicates were pooled, and the 95% confidence interval of  $k_{\text{obs}}$  was calculated from the standard error of the slope of the pooled data.

The second-order rate constant for inactivation by  $\text{NH}_3(\text{aq})$  ( $k_{\text{NH}_3}$  [ $\text{day}^{-1} \text{ liter mol}^{-1}$ ]) was determined by the best fit of a linear model according to the following relationship:

$$k_{\text{obs}} = k_{\text{NH}_3} \{\text{NH}_3(\text{aq})\} + k_{\text{background}}$$

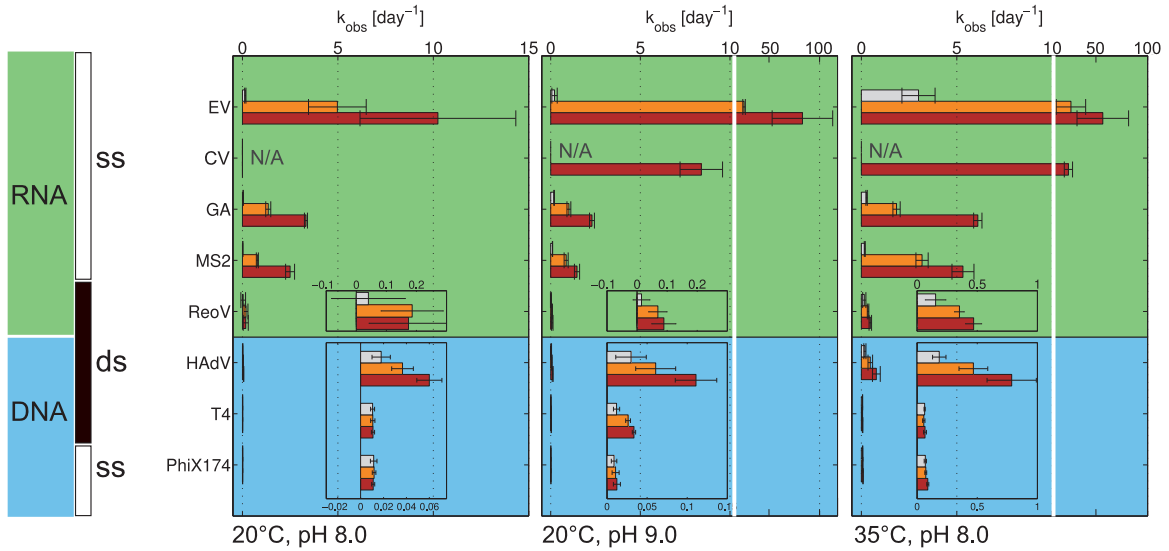
where  $\{\text{NH}_3(\text{aq})\}$  is the actual activity of  $\text{NH}_3(\text{aq})$  (listed in Table S1 in the supplemental material) and  $k_{\text{background}}$  is the first-order inactivation rate constant ( $\text{day}^{-1}$ ) in the absence of  $\text{NH}_3(\text{aq})$ .

Inactivation rate constants were compared by means of an analysis of covariance (ANCOVA) using the R software (47), applying a confidence threshold of 95%.

**DNA electrophoresis.** Electrophoresis experiments were performed to determine the dsDNA stability in the presence of  $\text{NH}_3(\text{aq})$  at mildly alkaline pH. Due to the high detection limit of the electrophoresis instrument, it was not possible to directly measure dsDNA genomes extracted from viruses. Instead, linearized plasmid DNA was used as our model dsDNA. Specifically, a concentrated stock of plasmid pBSK 22 (2,985 bp) was linearized with SpeI (lysis step: CutSmart buffer for 1 h at  $37^\circ\text{C}$  enzyme deactivation, 20 min at  $80^\circ\text{C}$ ; NEB reference no. R0133) and dephosphorylated with shrimp alkaline phosphatase (rSAP) (lysis step: CutSmart buffer for 30 min at  $37^\circ\text{C}$ ; enzyme deactivation, 5 min at  $65^\circ\text{C}$ ; NEB reference no. M0371). A  $50\text{-}\mu\text{l}$  volume of  $4 \mu\text{g liter}^{-1}$  plasmid solution was spiked into 10-ml serum flasks (Infochroma) containing VDB or AmCa (pH 9;  $\{\text{NH}_3(\text{aq})\}$ ,  $40 \text{ mmol liter}^{-1}$ ) at  $20^\circ\text{C}$ . Samples were periodically collected and stored at  $-20^\circ\text{C}$  until subjected to electrophoresis. Electrophoresis was carried out with an Agilent DNA high-sensitivity kit and a 2100 Bioanalyzer (Agilent Technologies, Inc.), according to the manufacturer's instructions.

## RESULTS

**Influence of  $\text{NH}_3(\text{aq})$  on virus inactivation kinetics.** All viruses studied exhibited a loss of infectivity with time, and inactivation

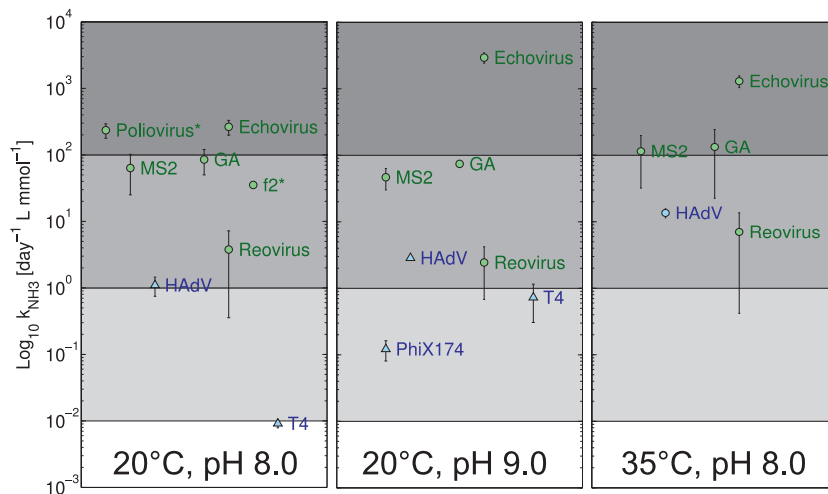


**FIG 1** Comparison of  $k_{obs}$  values for all viruses tested at pH 8/20°C, pH 9/20°C, and pH 8/35°C. The gray, orange, and brown bars correspond to intended  $\{NH_{3(aq)}\}$  of approximately 0, 20, and 40 mmol liter<sup>-1</sup>, respectively. The measured  $\{NH_{3(aq)}\}$  can be found in Table S1 in the supplemental material. The error bars depict the 95% confidence intervals associated with  $k_{obs}$  values. N/A, not available.

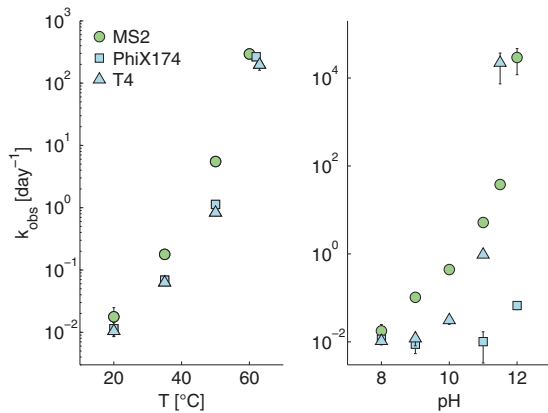
kinetics generally demonstrated a first-order model (see Fig. S1 and S2 in the supplemental material). Only for ReoV was poor adherence to first-order kinetics observed, in particular at pH 8/20°C, as well as at pH 9/20°C in PCa. **Figure 1** shows an overview of the corresponding first-order inactivation rate constants at pH 8 and 9 and at temperatures of 20 and 35°C. For each virus, the inactivation rate constant in ammonia-free PCa is shown, along with that in solutions with  $\{NH_{3(aq)}\}$  of approximately 20 and 40 mmol liter<sup>-1</sup>. In PCa, the  $k_{obs}$  of the different viruses were all within one order of magnitude (for exact values, see Tables S2 and S3 in the supplemental material). In the presence of  $NH_{3(aq)}$ , however, noticeable differences in inactivation were observed between the different viruses, which seemed to be correlated with genome type (**Fig. 1**). For ssRNA viruses, the presence of  $NH_{3(aq)}$  led to a

substantially faster inactivation, resulting in a 100- to 1,000-fold greater  $k_{obs}$  than that of viruses with other genome types.

The differing susceptibilities of viruses to  $NH_{3(aq)}$  as a function of genome type are further illustrated in **Fig. 2**, which shows the  $k_{NH_3}$  for the different viruses obtained at two pH values and temperatures. For ssRNA viruses, the  $k_{NH_3}$  was 10- to 1,000-fold higher than for viruses with other genome types under all conditions studied (for exact values of  $k_{NH_3}$ , see Table S4 in the supplemental material). The magnitude of  $k_{NH_3}$  reflects a virus' susceptibility to inactivation by  $NH_{3(aq)}$ . The large  $k_{NH_3}$  of ssRNA viruses thus indicates that  $NH_{3(aq)}$  affects ssRNA viruses more drastically than other viruses. Although  $k_{NH_3}$  was lower for DNA viruses, it was still significant for HAdV under all conditions tested and for  $\Phi X174$  and T4 at pH 9.



**FIG 2** Dependence of  $k_{NH_3}$  on temperature and pH. At pH 8 and 20°C, additional data for f2 and poliovirus (indicated by \*) were determined from the work of Burge et al. (57). The error bars depict the 95% confidence interval associated with  $k_{NH_3}$  values. Data points associated with the inactivation of  $\Phi X174$  and T4 at pH 8.0 and temperatures of 20 and 35°C that were not significantly different from zero were omitted (see Table S4 in the supplemental material).



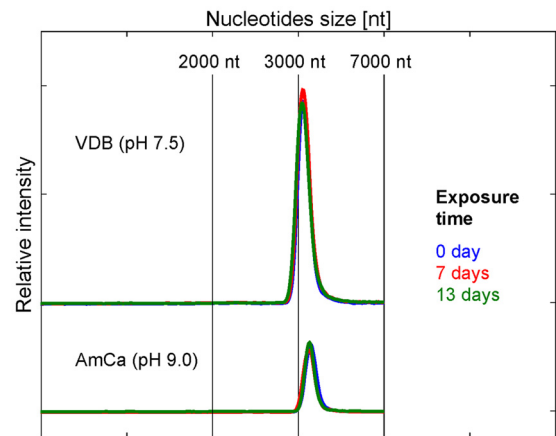
**FIG 3** Effect of temperature and pH on  $k_{\text{obs}}$  of MS2, T4, and  $\Phi$ X174. The effect of temperature was determined at pH 8 and the effect of pH at 20°C. The buffer composition can be found in Table S1 in the supplemental material. The error bars depict the 95% confidence intervals associated with  $k_{\text{obs}}$  values.

Differences in inactivation behavior were also observed within a single genome type, but they were small compared to the differences between genome types. For example, at 40 mM  $\text{NH}_3(\text{aq})$ , the differences in inactivation rate constants between genome types varied by a factor of 5 to 8,200 between ssRNA and dsDNA viruses, whereas among dsDNA viruses, inactivation varied by a factor of 3 to 10 and among ssRNA viruses by a factor of 1 to 50. Among the four ssRNA viruses studied, MS2 and GA showed significantly different  $k_{\text{obs}}$  ( $P < 0.05$ ) under all conditions tested, although they were of the same order of magnitude and exhibited the same trends upon changes in the solution conditions (Fig. 1; see also Table S2 in the supplemental material). EV exhibited a 4-fold greater  $k_{\text{obs}}$  than MS2 at pH 8/20°C in AmCa, and this difference was more pronounced when the temperature or pH was increased (Fig. 1). CV was also more readily inactivated than MS2 and GA, although it was more persistent than EV under all conditions tested.

**Effect of other solution conditions on inactivation. (i) pH.** The effect of pH on  $k_{\text{obs}}$  can be assessed by comparing the left and middle panels of Fig. 1. In the absence of  $\text{NH}_3(\text{aq})$ , no relevant increase in the  $k_{\text{obs}}$  of DNA viruses could be observed when raising the pH from 8 to 9. Among the RNA viruses studied, MS2 and GA exhibited an approximately 5-fold increase in  $k_{\text{obs}}$  (see Table S2 in the supplemental material). For EV and ReoV, the scatter in the data did not allow us to conclusively establish if the pH shift affected the  $k_{\text{obs}}$  (see Table S3 in the supplemental material). For three of the viruses studied (MS2,  $\Phi$ X174, and T4), inactivation was additionally quantified in PCa at pH values higher than 9. As shown in Fig. 3, increasing the pH to 12 resulted in a large effect on  $k_{\text{obs}}$  independent of the viral genome type.

Furthermore, the solution pH affected the susceptibilities of some viruses to  $\text{NH}_3(\text{aq})$ . Most notably, the dsDNA viruses HAdV and T4 exhibited a higher  $k_{\text{NH}_3}$  at pH 9 than at pH 8 (Fig. 2). Similarly, the  $k_{\text{NH}_3}$  of EV increased when raising the pH from 8 to 9. This indicates that for these viruses,  $k_{\text{NH}_3}$  is not a simple second-order rate constant. Instead, there appears to be a synergistic effect between hydroxide and  $\text{NH}_3(\text{aq})$  that promotes the inactivation of these viruses. For the other viruses tested, however,  $k_{\text{NH}_3}$  was independent of pH.

**(ii) Temperature.** A comparison of the left and right panels in



**FIG 4** Effect of  $\text{NH}_3(\text{aq})$  on dsDNA integrity. Electrophoresis measurement of linearized plasmid pBSK 22 (2,985 bp) after 0, 7, and 13 days in VDB and AmCa ( $\{\text{NH}_3(\text{aq})\} = 40 \text{ mmol liter}^{-1}$ ) at 20°C.

Fig. 1 and 2 illustrates the effect of temperature on  $k_{\text{obs}}$  and  $k_{\text{NH}_3}$ , respectively. As expected, an increase in temperature from 20 to 35°C led to greater  $k_{\text{obs}}$  and  $k_{\text{NH}_3}$  for all viruses tested (see Tables S2 to S4 in the supplemental material). However, DNA viruses showed a greater increase in  $k_{\text{obs}}$  (6- to 8-fold) than RNA viruses (2- to 4-fold), even though the absolute value of  $k_{\text{obs}}$  remained low for DNA viruses at 35°C, between 0.1 and 1 day<sup>-1</sup>. Three viruses (MS2,  $\Phi$ X174, and T4) were additionally tested at temperatures up to 60°C in PCa at pH 8 (Fig. 3), and they exhibited an exponential increase in  $k_{\text{obs}}$  with increasing temperature. MS2 was more sensitive to inactivation by temperature than the two DNA phages  $\Phi$ X174 and T4 up to 50°C. At 60°C, however, the gap between the  $k_{\text{obs}}$  of the three viruses narrowed.

**(iii) Ionic strength.** Increasing the ionic strength of PCa to match that of a 40 mM AmCa solution slightly enhanced the inactivation of the ssRNA viruses MS2, GA, and EV (see Tables S2 and S3 in the supplemental material). However, the effect of ionic strength on  $k_{\text{obs}}$  of ssRNA viruses remained negligible compared to the effect of  $\{\text{NH}_3(\text{aq})\}$ , confirming that  $\text{NH}_3(\text{aq})$ , not ionic strength, is the inactivating agent in AmCa. Among DNA viruses, an increase the ionic strength had either no effect on inactivation ( $\Phi$ X174) or lowered the inactivation rate constant (HAdV and T4).

**(iv) Monovalent cations.** Besides ionic strength, the specific effect of monovalent cations on the inactivation of EV was also tested, as cations, such as  $\text{K}^+$  or  $\text{NH}_4^+$ , were implicated in promoting genome degradation via the induction of nuclease activity of the virus capsid (48–50). At pH 9 and 20°C, no significant increase in  $k_{\text{obs}}$  could be observed with increasing  $\{\text{NH}_3(\text{aq})\}$ , whereas a comparable increase in  $\{\text{NH}_3(\text{aq})\}$  did enhance the  $k_{\text{obs}}$  (see Fig. S3 in the supplemental material).

**Effect of  $\text{NH}_3(\text{aq})$  on genomic material.** To elucidate the observed differences in the susceptibility of ssRNA and dsDNA viruses to  $\text{NH}_3(\text{aq})$  and to pH changes (Fig. 1), the effect of these two solution parameters on genomic material was investigated. Our previous work demonstrated that the ssRNA of MS2 readily degraded over the course of 3 days in the presence of  $\text{NH}_3(\text{aq})$  and upon increasing the pH from 7.5 to 9 (38). In contrast, as shown in Fig. 4, dsDNA remained intact in solutions containing  $\text{NH}_3(\text{aq})$  at pH 9.

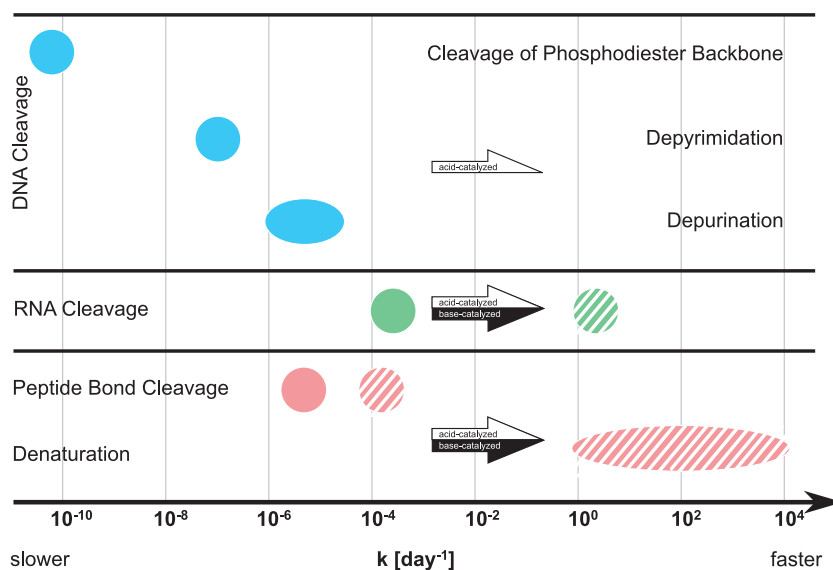


FIG 5 Summary and comparison of reported DNA, RNA, and protein degradation rate constants. Data refer to degradation at pH 7/37°C (filled symbols) and at pH 11/37°C (striped symbols), and were obtained from the literature (for references and exact numbers, see Table S5 in the supplemental material). The rate for spontaneous cleavage of the phosphodiester backbone corresponds to pH 7/25°C. The arrows indicate the direction of change of the rate constants upon changes in pH conditions (white for acid catalyzed, black for base catalyzed).

## DISCUSSION

In the following, we aim to explain the source heterogeneity in virus inactivation observed in this study. Specifically, this discussion focuses on the stability of the different viral components under conditions of HEAM storage and processing and the mechanisms contributing to their degradation. For this purpose, we draw on our experimental results, as well as on genome and protein degradation rate constants reported in the literature (compiled in Table S5 in the supplemental material and summarized in Fig. 5). Unfortunately, only few such degradation rates are published for viral genomes or proteins. We therefore included data in our analysis that are not specific to viruses. We acknowledge that reported genome and protein degradation rates vary widely, because they are inherently influenced by the specific structure and composition of the individual biomolecules studied. As such, they cannot be directly applied to any given virus. Despite this variation, the reported rates allowed us to establish a relative order of importance of the different degradation processes considered and thus were useful in establishing a general framework of the processes contributing to virus inactivation in HEAM.

**Causes underlying the differences in inactivation kinetics between DNA and RNA viruses.** The heterogeneous susceptibilities of viruses with different genome types to inactivation by  $\text{NH}_3(\text{aq})$  suggest that inactivation is associated with the stability of the different genome types in the presence of  $\text{NH}_3(\text{aq})$ . The observation that the variation among a single genome type was lower than the variation between genome types (e.g., ssRNA and DNA viruses) supports this hypothesis. Indeed, for ssRNA viruses, it has previously been shown that under the main range of temperatures (20 to 35°C), pH (8 to 9) and  $\{\text{NH}_3(\text{aq})\}$  conditions tested in this study, infectivity loss can mainly be related to genome degradation (38). In contrast, dsDNA exposed to  $\text{NH}_3(\text{aq})$  under the same conditions did not lose its integrity over the time period studied (Fig. 4). To rationalize this finding, the mechanisms involved in genome degradation by  $\text{NH}_3(\text{aq})$  must be considered.

For ssRNA phage MS2, genome degradation could be attributed to RNA cleavage via a general base-catalyzed transesterification (38), where the bases involved included  $\text{NH}_3(\text{aq})$ ,  $\text{OH}^-$ , and any other base in solution. In this process, the presence of the 2'-hydroxyl group of ribose renders the 3',5'-phosphodiester linkages of RNA molecules susceptible to base-catalyzed transesterification (51). This mechanism explains why ssRNA viruses are sensitive to changes in both pH and  $\text{NH}_3(\text{aq})$ , since both  $\text{OH}^-$  and  $\text{NH}_3(\text{aq})$  can act as the base catalyzing ssRNA transesterification. In contrast, the absence of the 2'-hydroxyl group in deoxyribose protects DNA from base-catalyzed transesterification (51). DNA cleavage can, however, spontaneously occur through the cleavage of the phosphodiester backbone, in particular at abasic sites (i.e., DNA sites lacking a purine or pyrimidine base) (52). The rate-limiting step in this process is acid catalyzed and involves the depurination, and to a lesser extent the depyrimidation, of a DNA base to form an abasic site. Except in the case of site-specific self-catalyzed depurination (53), the occurrence of which has not been studied for the DNA viruses considered herein, spontaneous depurination, and hence DNA cleavage, proceeds at an approximately 100-fold lower rate at pH 7.0 and 37°C than that with RNA cleavage (Fig. 5; see also Table S5 in the supplemental material). This lower rate of genome degradation explains why DNA viruses are more resistant to inactivation by  $\text{NH}_3(\text{aq})$  than RNA viruses under the conditions of this study (Fig. 1).

Besides genome degradation, virus inactivation can involve viral protein degradation through denaturation or peptide bond cleavage. To our knowledge, the degradation of viral proteins under typical HEAM conditions has not been studied. Furthermore, denaturation and peptide cleavage rates depend on protein composition and structure and can vary by orders of magnitude (see Table S5 in the supplemental material). Nevertheless, while not specific to viruses, reported peptide bond cleavage rates illustrate that this process is likely slower than ssRNA cleavage (Fig. 5). Similarly, around neutral pH, protein denaturation is not a rele-

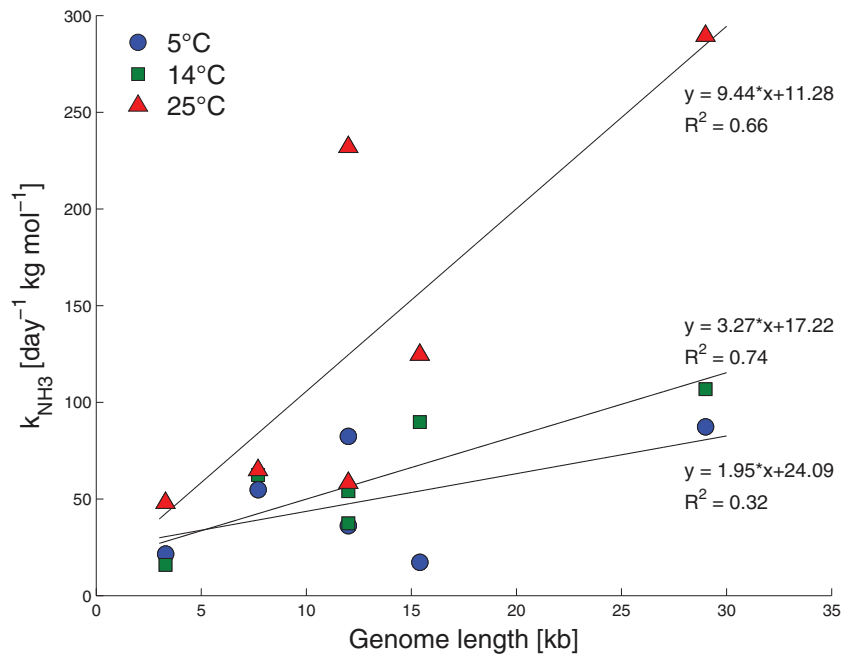


FIG 6  $k_{\text{NH}_3}$  for ssRNA viruses as a function of the genome length at different temperatures. Values for  $k_{\text{NH}_3}$  were calculated based on the  $k_{\text{obs}}$  reported by Emmoth et al. (17). The following genome lengths were assumed: MS2, 3.6 kb; feline calicivirus, 7.7 kb; avian influenza virus (H7N1 and H5N3), 12.0 kb; bovine parainfluenza virus type 3, 15.4 kb; and feline coronavirus, 29.0 kb. Note that the pH was not constant for given temperature conditions, and  $k_{\text{NH}_3}$  was determined from solutions with increasing pH (from 8.0 to 9.7). Furthermore, feline calicivirus, avian influenza virus, and bovine parainfluenza virus type 3 are enveloped viruses. Note that the concentration of  $\text{NH}_3$  was reported in moles per kilogram of treated hatchery waste.

vant process, because the protein folding rate is higher than the corresponding unfolding rate (see database produced by Bogatyreva et al. [54]). As a result, and as was established in Decrey et al. (38), protein modifications do not contribute significantly to the inactivation of ssRNA viruses under typical conditions of HEAM storage.

In the case of DNA viruses, in contrast, under conditions close to those investigated herein (pH 7.0 and 37°C), reported genome cleavage rates are similar to reported protein degradation rates (Fig. 5; see also Table S5 in the supplemental material). Thus, the inactivation of DNA viruses may involve both genome and protein destabilization under neutral pH conditions. However, the exact mechanism by which mildly alkaline conditions, and in particular  $\text{NH}_3(\text{aq})$ , promote DNA virus inactivation is currently not understood and warrants further investigation.

**Causes underlying differences in inactivation among RNA viruses.** Differences in inactivation kinetics were not only observed between DNA and RNA viruses but also among RNA viruses (Fig. 1 and 2). While these differences were small compared to those between ssRNA and DNA viruses, they were nevertheless statistically significant. A likely cause for this observation is the complex structure of ssRNA. Any ssRNA forms higher-ordered structures through base pairing and tertiary interactions (55). Among these structures, single-stranded regions are more likely to adopt in-line conformations, which are susceptible to cleavage by base-catalyzed transesterification (56). In comparison, highly structured and folded regions are locked into positions that are less accessible to attack by a base and therefore less likely to be cleaved. Thus, heterogeneity among ssRNA virus toward inactivation under the conditions of this study may be explained by the differences in sequence and length of the genome, which further

determine its structure. Specifically, the greater length of the EV and CV genomes (7,440 bases) compared to that of MS2 and GA (3,570 bases) may be in part responsible for their more rapid inactivation, as a longer genome implies a higher probability to form an in-line structure susceptible to cleavage. The differences in inactivation kinetics between short ssRNA genomes (MS2 and GA) and long ssRNA genomes (EV and CV) are consistent with reports by others. At pH 8/20°C, Burge et al. (57) observed similar differences between phage f2 and poliovirus (Fig. 2). Furthermore, the susceptibility of ssRNA viruses to  $\text{NH}_3(\text{aq})$  during the disinfection of hatchery waste was shown to be positively correlated with viral genome length (Fig. 6) (17). The genome length of EV, however, fails to explain why its sensitivity to  $\text{NH}_3(\text{aq})$  (and change in  $k_{\text{NH}_3}$ ) increases so drastically compared to MS2, GA, and CV upon increasing the pH from 8 to 9 or upon raising the temperature from 20 to 35°C (Fig. 1 and 2). These strong increases in  $k_{\text{NH}_3}$  imply that additional factors, possibly linked to specific capsid features, promote the virucidal action of  $\text{NH}_3(\text{aq})$  on EV. As discussed by Decrey et al. (38), monovalent cations, such as  $\text{NH}_4^+$  or  $\text{K}^+$ , have been postulated to enhance endonuclease activity exerted by the viral capsid, causing additional genome degradation. However, this process could be confirmed neither for MS2 (38) nor for EV, as  $\text{K}^+$  failed to promote inactivation (see Fig. S3 in the supplemental material). The reason for the great sensitivity of EV toward  $\text{NH}_3(\text{aq})$  thus remains unknown.

Unlike DNA viruses, which did not exhibit significant differences in inactivation whether they were single or double stranded, the dsRNA ReoV exhibited higher resistance against  $\text{NH}_3(\text{aq})$  inactivation than ssRNA viruses. Similar trends were reported by others, including Ward and Ashley (19) and Makaya et al. (58), who also observed that ReoV was more resistant than other ssRNA

viruses (polio-, coxsackie-, and echovirus) in anaerobically digested sludge, and bovine rotavirus A was more resistant than murine norovirus in stored urine, respectively. A likely explanation for the resistance of dsRNA to  $\text{NH}_{3(\text{aq})}$  lies in its configuration. Usher (59) suggested that the 5'-oxygen of any internal nucleotide unit of a RNA double helix is unlikely to take on an in-line conformation conducive to cleavage by base-catalyzed transesterification (56). This was later experimentally confirmed by observations of single-stranded RNA, in which folded domains with double-stranded configuration were more resistant to cleavage than single-stranded regions (60–62). Finally, Burge et al. (57) suggested that cleavage of double-stranded genomes is slower because the double strands would require two chains to rupture to be cleaved.

No relevant effect of strand conformation could be observed in the case of DNA viruses, likely because the cleavage process was very slow compared to that of RNA viruses.

**Inactivation mechanisms under extreme pH and temperature conditions.** One way to enhance virus inactivation in HEAM is to push the system toward even harsher conditions of pH and temperature than those naturally encountered during storage. Under such extreme conditions, the inactivation kinetics of ssRNA and DNA viruses no longer exhibited major differences, with the notable exception of  $\Phi\text{X174}$  at pH 12 (Fig. 3). It was also shown that in lime-stabilized biosolids at pH 11.5 to 12.0 and temperatures of 4 and 28°C, differences in inactivation were rather low for MS2, reovirus, and hepatitis A (29) and for MS2, rotavirus, and adenovirus (63). A shift to more-alkaline conditions enhances RNA and peptide bond cleavage, as well as protein denaturation, which are base-catalyzed processes (64–69), but not the rate-limiting step of DNA cleavage, depurination, which is an acid-catalyzed process (70) (Fig. 5). Thus, when increasing the pH, the rate of protein degradation, in particular denaturation, will be similar to or higher than that of RNA cleavage and much higher than that of DNA cleavage (Fig. 5). Therefore, RNA and DNA virus inactivation should shift from only genome- and genome/protein-mediated inactivation, respectively, to mostly protein-mediated inactivation under more-alkaline conditions. We thus hypothesize that protein denaturation is the main mechanism responsible for the inactivation of all viruses under highly alkaline pH conditions.

While reported genome and protein degradation rates for high temperature were not available, several studies present evidence for protein denaturation as the main inactivation mechanism at temperatures characteristic of thermophilic HEAM digestion. Aitken et al. (71) observed a high activation energy characteristic of protein denaturation for poliovirus inactivation in biosolids under thermophilic anaerobic conditions (49 to 53°C). Nuanualsuwan and Cliver (72) reported that the primary target of poliovirus, hepatitis A virus, and feline calicivirus inactivation at 72°C was the capsid, and that inactivation occurred by conformational change of the viral proteins. Similarly, Romero-Maraccini et al. (73) proposed that rotavirus inactivation at 57°C was linked to a disruption of protein-mediated steps of the early life cycle. Finally, the virucidal mode of action of high temperature and pH may also involve a denaturation of the viral capsid proteins that allows for the release of the genome from the capsid. Genome release was found to occur at a pH between 8 and 9 for an ssRNA insect virus (74) and at a temperature of 45 to 70°C at pH 7.0 for dsDNA phage  $\lambda$  and HK97 (75, 76) and ssRNA poliovirus, bovine enterovirus,

and equine rhinitis A virus (77, 78). Specifically, Duda et al. (75) reported a rate constant of around  $10^2 \text{ day}^{-1}$  for the release of the HK97 genome from capsid at 65°C, which is the same order of magnitude than the  $k_{\text{obs}}$  for phages at 60°C in this study (Fig. 3; see also Table S2 in the supplemental material).

Whether protein degradation causes inactivation through loss of host attachment, genome release, or a combination of these viral functions remains unclear and is likely virus dependent. However, it can be concluded that inactivation under extreme conditions appears to be protein related and therefore independent of genome type. While our data as well as those of other studies (34, 79) thus showed a reduction in the heterogeneity among viruses under extreme conditions, other works have demonstrated that some heterogeneity in virus inactivation kinetics is conserved even under high-pH and -temperature conditions (29, 57, 63, 80, 81). Very resistant viruses include phage  $\lambda$  (82) and  $\Phi\text{X174}$  (this study) for high pH and parvovirus and *Salmonella* phage 28B for high temperature (30, 83–86). The reasons for these resistant behaviors remain unknown.

**Implication of this study for virus inactivation in HEAM.** This work suggests that the heterogeneity in virus inactivation kinetics in HEAM is correlated with genome type in neutral to mildly alkaline conditions and at temperatures below 50°C. Under more-extreme conditions of pH and temperature, however, inactivation is associated with a loss of protein structure and stability. Thus, for the subset of viruses studied herein, virus genome type is of less importance during alkaline stabilization or thermophilic digestion, whereas it is a key parameter in determining virus fate during HEAM storage or mesophilic digestion. While our results remain to be confirmed for additional viruses, we suggest, based on the basic genome and protein degradation mechanisms considered herein, that DNA and dsRNA viruses, e.g., adenovirus and rotavirus, respectively, are the most persistent during storage or mesophilic digestion. Consistent with this notion, a recent survey of urine storage tanks in Durban (South Africa) revealed that JC polyomavirus (dsDNA), adenovirus, and rotavirus were frequently detected, whereas ssRNA virus, such as norovirus, hepatitis A virus, astrovirus, and enterovirus, were only rarely found (87).

The kinetic trends observed in this study were generally consistent with studies using real matrices (19, 26, 30, 34–36, 58), although some works reported contradictory results (25, 27, 32, 37). This may be due to the high complexity of real matrices compared to the well-controlled solutions used in this study. Real matrices, like sludge or urine, however, may contain additional inactivating factors than the ones considered herein. For example, the presence of metal ions may enhance inactivation, especially in the case of ssRNA virus, by accelerating RNA base-catalyzed transesterification (66). Detergents were also shown to reduce and increase the inactivation of enterovirus and reovirus, respectively (88). In the case of sludge, viruses may adsorb to the solid fraction and thereby be protected from the inactivating agents present in the bulk (89). Furthermore, the water content of sludge can be an important parameter, as it determines the mobility and concentration of inactivating or protective agents, and water evaporation itself can lead to virus inactivation during the dewatering process (90–92). Finally, microbial or related enzyme activity may also enhance inactivation during sludge digestion (93–96).



The conclusions of this study were drawn from experiments with naked viruses, which constitute the main fraction of enteric viruses. However, enveloped viruses, such as coronaviruses, can also be excreted in HEAM (97), and they are expected to behave differently due to the instability of the lipid bilayer under alkaline conditions. It was observed that phospholipids were cleaved at a rate of  $\sim 1 \times 10^{-2} \text{ day}^{-1}$  at 40°C and pH 9 (98), which is close to the RNA cleavage rate constant for similar conditions ( $1.6 \times 10^{-2} \text{ day}^{-1}$  at pH 9 and 37°C) (65). Consequently, Elving et al. (99) showed that MS2 was more persistent than the enveloped dsRNA phage  $\Phi 6$  and the enveloped ssRNA influenza A virus during composting of manure at 35 to 55°C. Similarly, Ye et al. determined that, independent of genome type, enveloped viruses (ssRNA murine hepatitis virus and dsRNA phage  $\Phi 6$ ) were inactivated at higher rates than naked viruses (MS2 and dsDNA phage T3) in wastewater (100). In contrast, Emmoth et al. (17) did not observe a significant influence of the envelope among ssRNA virus inactivated in manure at 4 to 25°C.

Concerns about fecal-oral transmission of viruses responsible for recent outbreaks, such as coronavirus (severe acute respiratory syndrome [SARS]) (101, 102) or filovirus (Ebola virus) (103), may be partially quelled from the results of this study. The facts that they are ssRNA viruses and possess an envelope make them very sensitive to inactivation in human excreta during simple treatment, such as storage or mesophilic digestion, if mildly alkaline conditions with substantial amount of  $\text{NH}_3(\text{aq})$  are reached.

Finally, based on the results of this study and work by others referenced herein, we can issue recommendations regarding the use of indicators for the inactivation of actual human viruses in HEAM. Consistent with the suggestion by Emmoth et al. (17), we propose the use of MS2 as a conservative indicator to assess the inactivation of ssRNA viruses in HEAM treated under mildly alkaline conditions (pH 7.0 to 9.5) and at moderate temperatures (4 to 35°C). The very stable  $\Phi X174$  or dsDNA phages (T phages, PRD1, or *Salmonella* phage 28B) can be used as indicators for persistent viruses. Under higher pH and temperature conditions, however, the use of very resistant viruses, such as  $\Phi X174$  or parvovirus, may overestimate treatment requirements.

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