Virus inactivation mechanisms in human urine and fecal sludge

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Abstract

Water, sanitation and hygiene interventions are among the most significant health interventions addressing the worldwide burden of diarrheal disease and environmental enteropathy. Sanitizing human excreta and animal manure (HEAM) is a critical step in reducing the spread of disease and ensuring microbially safe reuse of waste materials. From the perspective of human excreta, it was recently established that despite an increase in global toilet coverage, the proportion of safely managed fecal waste remains low. Therefore, on-site storage and treatment still represent the best opportunities to reduce pathogen load. Among these pathogens, viruses are particularly persistent. However, adequate storage or digestion of HEAM can strongly reduce the number of viruses by creating adverse conditions to their survival. Although temperature, pH and ammonia (NH_3) are commonly reported as primary virus inactivation factors, the mechanisms underlying virus reduction remain unclear. This thesis aims to shed more light on these mechanisms and their exploitation for HEAM management.

Unlike other living organisms, viruses carry their genetic information under different forms, specifically as single- (ss) or double-stranded (ds) RNA or DNA. ssRNA viruses were shown to be the most sensitive virus type during HEAM treatment. Additionally, ssRNA is also the most common genome type among enteric viruses. Therefore, we first investigated the kinetics and mechanisms of inactivation of the ssRNA virus MS2 under temperature, pH and NH_3 conditions representative of waste storage. MS2 inactivation was mainly controlled by the activity of NH_3 over a pH range of 7.0 to 9.5 and temperatures lower than 40°C. Other bases (e.g., hydroxide, carbonate) additionally contributed to the observed reduction of infective MS2. The loss in MS2 infectivity could be rationalized by a loss in genome integrity, which was attributed to genome cleavage via general base-catalyzed transesterification. The presence of the 2'-hydroxyl group of ribose renders the 3',5'-phosphodiester linkages of RNA molecules susceptible to base-catalyzed transesterification. The contribution of each base to genome transesterification, and hence inactivation, could be related to the pK_a of the conjugated acid of the base by means of a Brønsted relationship. Based on the Brønsted relationship in conjunction with the activity of bases in solution, a model was formulated that enabled an accurate prediction of MS2 inactivation rates in synthetic solutions.

Several studies have observed slower inactivation in HEAM among viruses with genome types other than ssRNA. The reasons for this enhanced persistence, however, are not clear. Here, we systematically investigated the inactivation of viruses with different genome type in synthetic solutions with well-controlled temperature, pH and NH_3 conditions representative of waste

storage. A total of seven viruses representing all genome types were studied: bacteriophages MS2 and GA and human echovirus (ssRNA); mammalian reovirus (dsRNA); bacteriophage ΦX174 (ssDNA); and bacteriophage T4 and human adenovirus (dsDNA). DNA and dsRNA viruses were confirmed to be considerably more resistant than ssRNA viruses. The causes underlying the differences in inactivation kinetics were, in the case of DNA viruses, the absence of the 2'-hydroxyl group in deoxyribose, which protects DNA from cleavage by base-catalyzed transesterification. In the case of dsRNA viruses, the geometry of a RNA double helix prevents a structural conformation conducive to cleavage by base-catalyzed transesterification. Notably, pushing the system toward even harsher pH and temperature 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-related inactivation mechanisms common to all viruses. Thus, under conditions encountered during thermophilic digestion and alkaline stabilization, the most probable mechanism driving virus inactivation is the disruption of protein integrity. This may lead to different inactivating events, such as loss of host attachment, inhibition of genome delivery or genome release from the capsid.

The observations made in synthetic solutions under controlled conditions helped to distinguish between different factors governing virus inactivation. However, such well-controlled systems oversimplify the complexity encountered in real matrices, such as urine, fecal sludge or animal manure. Therefore, in the last chapter of this thesis, the principles of virus inactivation established in well-controlled systems were verified in real waste matrices. For that purpose, the inactivation kinetics of the base-sensitive ssRNA phage MS2 as well as the more resistant DNA phages T4, ΦX174 and the dsDNA human adenovirus were determined in different batches of stored urine and sludge. The predictive model of MS2 inactivation established for synthetic solutions was shown to generally apply reasonably well to inactivation in real matrices. In diluted stored urine, however, the model underestimated measured inactivation. This could be explained by the presence of metal ions that are not accounted for in the model, and that promote the transesterification of the ssRNA genome of MS2. The differences in inactivation kinetics observed in synthetic solutions among virus with different genome types were generally conserved in stored urine and sludge. However, human adenovirus showed similar to higher sensitivity than MS2 to stored urine and sludge. This was attributed to adenovirus' sensitivity to microbial or enzymatic activity encountered in urine and sludge.

Overall, this work provides a better comprehension of the mechanisms underlying virus inactivation during human excreta and animal manure treatment. It allowed establishing a comprehensive prediction model for MS2 inactivation in HEAM, which is made available on the internet. Finally, this thesis offers a simple decision making tool to optimize virus inactivation during storage and treatment of human excreta and animal manure.

Key words: Virus inactivation, ammonia, stored urine, feces, sludge, base-catalyzed transesterification, genome type, storage, mesophilic/thermophilic digestion, alkaline stabilisation, adenovirus, echovirus, reovirus, phages, microbial activity.

Résumé

Les mesures d'hygiène, d'assainissement et de traitement de l'eau font partie des interventions les plus efficaces permettant de combattre les infections gastro-intestinales. Celles-ci peuvent se déclarer de manière aigüe (diarrhée) ou sous forme chronique et représentent une réelle menace particulièrement pour la santé des enfants en bas-âge dans les pays où les systèmes de santé et les conditions de vie sont précaires. Assainir les excréments humains et le lisier animal (EHLA) est, dès lors, une étape critique dans la réduction de la transmission de ces infections ainsi que dans l'optique d'une réutilisation, par exemple comme fertilisant dans l'agriculture. Dans le cas des excréments humains, il a été récemment établi que, malgré une augmentation mondiale de l'accès aux toilettes, la proportion d'excréments correctement prise en charge et traitée restait très basse. Par conséquent, le stockage et d'éventuels traitements dits sur site se révèlent être les meilleures opportunités offertes pour réduire la charge en agent pathogène des excréments. Parmi ces agents pathogènes figurent les virus qui sont particulièrement persistants. Cependant, un stockage ou une digestion microbienne appropriée d'EHLA permet de réduire fortement le nombre de ces virus par l'intermédiaire de la formation de conditions défavorables à leur survie. Bien que la température, le pH et l'ammoniac (NH_3) soient communément rapportés comme des facteurs prépondérants dans l'inactivation de virus, les mécanismes y étant impliqués restent peu clairs. Cette thèse a pour objectif de mettre en lumière ces mécanismes et la manière dont ils peuvent être exploités lors de la prise en charge et le traitement d'EHLA.

Contrairement à d'autres organismes vivants, les virus portent leur information génétique sous différentes formes, ARN ou ADN, simple (ss) ou double brins (ds). Les virus ssARN se montrent généralement être le type de virus le plus sensible aux traitements d'EHLA. De plus, le génome ssARN représente le genre dominant parmi les virus responsables d'infections gastro-intestinales. Par conséquent, nous avons d'abord concentré notre étude sur les cinétiques et mécanismes causant l'inactivation du phage ssARN MS2 dans des conditions de température, pH et NH_3 représentatives du stockage d'EHLA. L'inactivation de MS2 était principalement contrôlée par l'activité de NH_3 pour un domaine de pH entre 7.0 et 9.5 et des températures inférieures à 40°C. De manière additive, d'autres bases tels que l'ion hydroxyde et les carbonates ont contribué à l'inactivation de MS2. La perte des propriétés infectieuses de MS2 a pu être assimilée à la perte d'intégrité de son génome attribuée au clivage par un mécanisme appelé transestérification générale catalysée par des bases. En effet, la présence d'un groupement hydroxyle en position 2' sur le ribose de l'ARN rend les liaisons phosphodiester

entre 3' et 5' de l'ARN susceptibles au mécanisme susmentionné. La contribution de chaque base au clivage du génome de MS2, et par ce biais à son inactivation, a pu être reliée au pK_a de l'acide conjugué de celle-ci au travers d'une relation dite « de Brønsted ». Sur cette base et conjointement à l'activité mesurée de chaque base présente en solution, il a été possible de formuler un modèle permettant une estimation précise de l'inactivation de MS2 dans des solutions de synthèse.

Plusieurs études ont déjà décrit des cinétiques d'inactivation plus lentes que celles des virus ssARN pour des virus possédant d'autres types de génome. Les raisons de cette remarquable résistance demeurent cependant peu claires. Dans ce travail, nous avons comparé de manière systématique l'inactivation de virus possédant des génomes différents dans des solutions de synthèse sous des conditions contrôlées de température, pH et NH_3 représentatives du stockage d'EHLA. Un total de sept virus représentant l'ensemble des types de génome existants ont été étudiés : les phages MS2 et GA et le virus humain echovirus (ssARN) ; le virus de mammifère reovirus (dsARN) ; le phage ΦX174 (ssADN) ; et le phage T4 et le virus humain adenovirus (dsADN). Les virus ADN et dsARN se sont effectivement avérés considérablement plus résistants que les virus ssARN. Dans le cas des virus ADN, la cause sous-jacente à cette différence était l'absence d'un groupement hydroxyle en position 2' sur le déoxyribose de l'ADN. Ceci a eu pour effet de protéger l'ADN contre le clivage dû à la transestérification catalysée par des bases. Pour ce qui est des virus dsARN, la géométrie du double brin a permis d'empêcher le génome de se trouver dans une conformation moléculaire conduisant au clivage par transestérification catalysée par des bases. De manière remarquable, repousser les limites de notre système vers des conditions de température et de pH plus rudes, telles qu'envisagées durant une digestion microbienne thermophile ou une stabilisation à l'aide d'agents alcalins tels que la chaux, a eu comme tendance l'élimination des disparités entre les types de virus observées plus haut. Ceci suggère que la dépendance de l'inactivation virale au type de génome en condition de stockage disparaît au profit de mécanismes liés aux protéines virales communs à tous les virus dans le cas d'une digestion microbienne thermophile ou d'une stabilisation à l'aide d'agents alcalins.

Les observations faites dans des solutions synthétiques en milieux contrôlés ont permis de distinguer les facteurs primordiaux régulant l'inactivation des virus. Cependant, ces mêmes solutions simplifient la complexité des matrices réelles que sont l'urine, les matières fécales et le lisier. Par conséquent, dans le dernier chapitre de cette thèse, il nous a fallu vérifier les conclusions préalablement tirées en milieux complexes. Pour ce faire, les cinétiques d'inactivation du ssARN phage MS2 vulnérable aux bases et de ses plus coriaces camarades ADN (les phages T4 et ΦX174 ainsi que le virus humain adenovirus) ont été déterminées et comparées dans différentes urines et boues fécales. Le modèle établi dans des solutions synthétiques, permettant la prédiction de l'inactivation de MS2, s'est avéré raisonnablement fiable dans les matrices testées. Cependant, dans l'urine fortement diluée, les prédictions ont eu tendance à sous-estimer l'inactivation observée. Ceci pourrait être expliqué par la présence de cations métalliques en faible concentration, dont notre modèle ne tient pas compte, mais qui peuvent promouvoir la réaction de transestérification du génome ssARN. Les grandes différences

d'inactivation entre types de virus observées dans les solutions synthétiques se sont révélées généralement conservées en solution réelle. Néanmoins, et il faut avouer « avoir été surpris en bien », le virus humain adenovirus a démontré être aussi, voire plus, sensible que le phage ssARN MS2 dans ces conditions. Il semblerait que l'activité microbienne ou enzymatique puisse être la raison de la grande sensibilité d'adenovirus.

De manière globale, cette thèse procure une compréhension détaillée des mécanismes gouvernant l'inactivation des virus dans les excréments humains et le lisier animal. De plus, elle fournit un modèle simple de prédiction de l'inactivation de MS2 dans des conditions de stockage gratuitement disponible sur internet. Finalement, ce travail offre un outil simple d'aide à la décision dans le suivi et l'amélioration de l'inactivation des virus durant le stockage et le traitement des EHLA.

Mots clefs : Inactivation de virus, ammoniac, urine, fèces, boues, transestérification catalysée par des bases, type de génome, stockage, digestion mésophile et thermophile, stabilisation par agents alcalins, activité microbienne, adenovirus, echovirus, reovirus, phages.

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List of abbreviations

AmCa - ammonium carbonate buffer
DNA - deoxyribonucleic acid
ds - double-stranded
EC - electrical conductivity
EV - echovirus
FBS - fetal bovine serum
HAdV - human adenovirus
HEAM - human excreta and animal manure
IS - ionic strength
kb - kilo-base
KCa - potassium carbonate buffer
MEAct - microbial and enzymatic activity
MPNCU - most probable number of cytopathogenic unit
NH3 - uncharged form of ammonia
nt - nucleotide
PCa - phosphate carbonate buffer
PFU - plaque forming unit
ReoV - reovirus
RNA - ribonucleic acid
RT-qPCR - reverse transcription quantitative polymerase chain reaction
SCOD - soluble chemical oxygen demand
ss - single-stranded
T - temperature
TIC - total inorganic carbon
TS - total solids
VDB - virus dilution buffer
WASH - water, sanitation and hygiene

1 Introduction

1.1 Context

Water, sanitation, hygiene and human health. Despite advances in technology and healthcare since the 19th century, the mortality and morbidity rates resulting from diseases associated with poor water, sanitation and hygiene (WASH) conditions remain considerable. The magnitude of the challenge to human health caused by these diseases, and the opportunity for addressing it through improvements in WASH, is illustrated by the following facts, as summarized by Bartram et al.[1]:

- The WASH-associated disease burden is dominated by mortality from infectious diarrhea, nearly 90% of which is borne by children under five years old and 73% of which occurs in only 15 developing countries.
- Diarrhea alone kills more young children each year than HIV/AIDS, tuberculosis and malaria combined, and the key to controlling it is WASH.
- In 2011, an estimated 36 million episodes of diarrhea and 18 million episodes of pneumonia progressed to severe episodes, including 700,000 and 1.3 million deaths, respectively.[2]
- Around 2.4 million deaths (4.2% of all deaths), mostly children in developing countries, could be prevented annually if everyone practiced appropriate hygiene and had access to adequate sanitation and drinking water.
- 1.1 billion people habitually defecate in the open, whereas 4.1 billion people have some form of improved sanitation.

- Adequate sanitation can not only prevent endemic diarrhea, but also help to prevent intestinal helminthiases,[3] giardiasis, schistosomiasis, trachoma and other enteric infections.

From a global point of view, however, the relative contribution of WASH-associated illness can appear to be rather low. A recent global study on the risk factors for burden of disease ranked the factors of "Unimproved sanitation" and "Water" in 25th and 34th position, respectively.[4] Furthermore, it was estimated that even together, unimproved access to water and sanitation accounted for 2.1% and 0.9% of the global disease burden in 1990 and 2010, respectively.[4] Schmidt[5] suggests a careful reading of these numbers, especially due to the way "unimproved" was defined and the difficulty in quantifying long-term, indirect health benefits. In addition to this, the top risk factors can vary geographically: in the sub-Saharan region of Africa, unimproved water and sanitation were among the top ten risk factors accounting for the burden of disease. Thus, WASH interventions have the potential to make a huge impact on improving human health in targeted locations such as sub-Saharan Africa.

The global impact of WASH interventions can be estimated based on national statistics. The most recent studies by the World Bank (Water and Sanitation program unit) were conducted on a large scale in an attempt to establish causative links between economic (e.g., adult wages), health (e.g., infant mortality, height, cognitive skills) and sanitation variables (e.g., open defecation).[6–8] These works demonstrated statistically relevant associations between: open defecation and child height;[6] being born in a district or calendar year with lower infant mortality and better sanitation and earning higher wages;[8] and being exposed to a sanitation campaign and improved average cognitive skills[7] Although these global associations are statistically relevant, they need to be confirmed in the field by testing whether WASH interventions actually have an impact on human health and wealth outcomes at the household level.

The evaluation of local WASH interventions on diarrhea reduction generally shows a positive impact.[9,10] However, the magnitude of this effect is heterogeneous: from no reduction in diarrheal disease to more than an 80% reduction.[9,11] Furthermore, evaluations of WASH interventions are largely prone to publication bias, lack of blinding, and lack of randomization, which are due especially to the severe constraints of implementing such interventions.[5,11,12] Even the largest observed impacts can sometimes be explained by bias.[5] A recently updated meta-analysis of water supply and sanitation interventions suggested that their impact on diarrheal disease reduction was much smaller than previously thought.[13] Thus, as suggested by Schmidt,[5] reliable data on the health impacts of WASH interventions at the local scale are lacking, and new research methods could help to provide better evidence.

Recent advances in molecular biology allow the identification of sources of pathogens in the field using conventional or molecular methods. This technique helps us to understand pathogen transmission pathways and further evaluate the impact of these different pathways on the prevalence of diarrhea.[14–16] The fecal-oral pathogens found in human excreta and animal manure (HEAM) are transmitted to humans via a range of pathways, as illustrated in

Figure 1.1. While this standard diagram is well-accepted for helping us to understand the role of pathogens in disease transmission, the variability of pathogen characteristics and field site conditions render the reality more complex. For example, pathogen detection using molecular methods, on human hands and in source and stored water, showed the presence of viral and bacterial pathogens in all types of samples. However, viruses were more frequently detected on hands, whereas bacteria were consistently reported in all types of samples, suggesting that hands are an important vector for viral contamination.[14] At a household level, Pickering et al. observed a correlation between fecal contamination on hands and fecal contamination in stored drinking water as well as with the prevalence of gastrointestinal disease.[15] Mattioli et al., however, noticed an association between the decreasing chances of a child aged under five having reported diarrhea and the detection of E.coli virulence gene on its hands and in stored water.[16] These two examples illustrate the complexity of relating pathogen occurrence to diarrheal prevalence, as well as the variability that should be expected between site-specific studies.

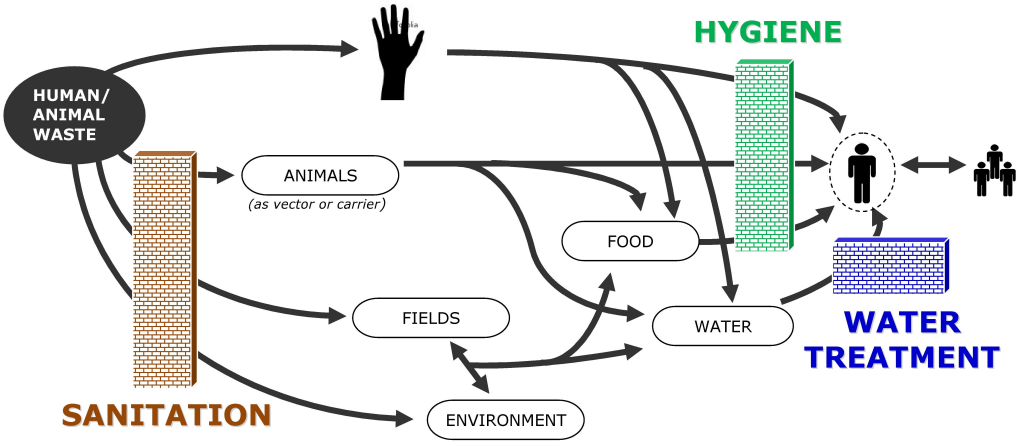


Figure 1.1: *Diagram of main pathogen transmission pathways and WASH interventions.*

The etiology of diarrhea in young infants is also a key parameter in better understanding and designing interventions in the field. Kotloff et al. observed that most attributable cases of moderate-to-severe diarrhea in children aged 0-59 months residing in censused populations at four sites in Africa and three in Asia were due to four pathogens: rotavirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* producing heat-stable toxin and *Shigella*.[17] Taniuchi et al. showed that diarrheal events in Bangladeshi infants, from resource-limited settings and in their first year of life, were associated with a state of overall pathogen excess.[18] Using molecular methods, an average of 5.6 and 4.3 enteropathogens were detected in stool samples from infants with and without diarrhea, respectively. Typically, one excess pathogen was detected, leading to the diarrheal event. For bacteria, this pathogen was frequently either Campylobacter or entero-aggregative or entero-pathogenic E.coli. The excess protozoan was

frequently Entamoeba, and the excess virus was frequently rotavirus. For infants in the United States, one or fewer enteropathogens were detected in stool samples, both in control cases (healthy infants) and during diarrheal episodes.[18] These results illustrate that:[19–21] 1. interventions to control a single pathogen in resource-limited settings may have limited impact; 2. even though death rates from diarrhea are being reduced worldwide, the repercussions on morbidity associated with a polypathogen mix in the intestine of surviving infants and children living in resource-limited settings should not be neglected. A chronic state of intestinal inflammation (also called "environmental enteropathy") is associated with intestinal leakage and malabsorption, leading to malnutrition and micronutrient deficiencies.

As illustrated above and stated by Schmidt,[5] the observable effects of WASH on the global burden of disease remain elusive. Whereas it is commonly admitted that WASH fundamentally improves people's lives, wellbeing and health in poor settings, epidemiological research may simply not be the right tool to prove this. The topic's complexity might explain why scientific studies consistently fail to show robust evidence of causal relationships between WASH interventions and health improvements. Nevertheless, WASH interventions are still amongst the most significant health interventions addressing the burden of diarrheal disease and environmental enteropathy.[19] These health challenges are attributable to a heterogeneous group of pathogens[18] and, therefore, difficult to tackle using vaccination or medication. In the short-term, the non-health benefits of WASH interventions (e.g., educational, developmental, gender-related, environmental and resource benefits) are more likely to be recognized by those individuals affected.[22] These short-term improvements can, therefore, be seen as the major driving forces behind the implementation of WASH interventions, all of which will benefit human health in the long-term (Figure 1.2).

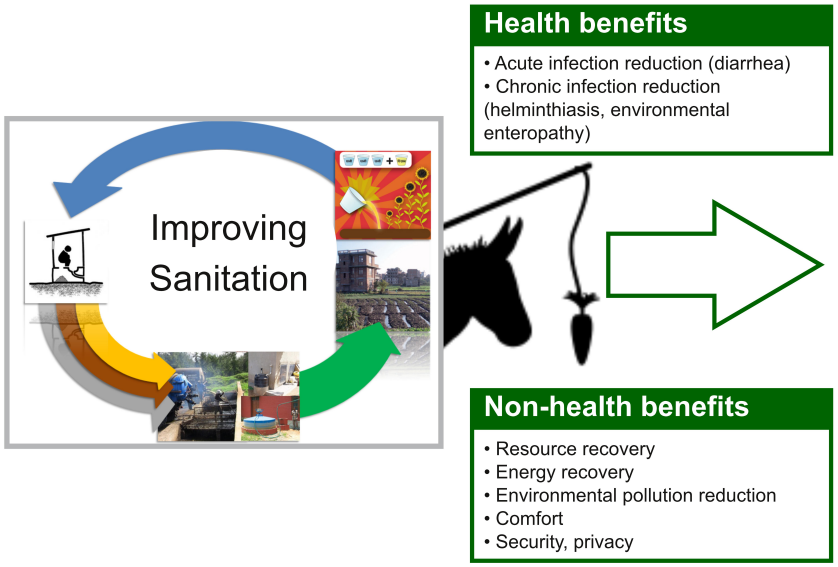


Figure 1.2: *The driving forces leading to the implementation and improvement of sanitation.*

Resource scarcity and environmental pollution. Among the non-health benefits of WASH interventions, reducing environmental pollution and the chance of related resource-recovery are especially important driving forces for improving sanitation interventions. HEAM can contain nutrients, pharmaceuticals[23] and heavy metals[24] and, therefore, adequate pre-treatment is necessary to prevent environmental contamination. Indeed, phosphorus (P), potassium (K) and sulfur (S) are not only non-renewable resources, but also macronutrients essential in agricultural production and for many industrial processes. In contrast to natural cycles, where most of these nutrients are used parsimoniously and recycled in a self-sustaining system, humans extract these resources from the earth's crust and exploit them with abandon, often without thought of saving or recycling. This practice inevitably leads to long-term depletion. For example, it has been predicted that our society will run out of P by the end of this century.[25] Although the P in HEAM represents only a small fraction of global P flows, major reductions in the demand for P fertilizer can be achieved by improving plant nutrition management and recycling of HEAM. For their part, Larsen et al. concluded that nitrogen (N) and P from human metabolisms are of negligible importance to the global N cycle and of minor importance to the global P cycle, however, they are of extreme importance to coastal water and freshwater pollution.[26] Finally, it should be mentioned that resources are not equally distributed around the world, and the recycling of locally available resources such as HEAM can be crucial in locations where farmers can barely afford mineral fertilizers.[27]

Towards ecological and sustainable sanitation. Sanitation has a relatively broad beneficial impact on health and non-health factors (Figure 1.2). Similar to water and hygiene interventions, sanitation measures are mainly aimed at reducing the health and environmental impact of human excreta. However, these driving forces are often not enough to overcome the high cost of sanitation facilities or treatment plants. At the household scale, a simple example involving the construction of private latrines in Benin illustrates that safety (against animals and the dangers of night-time defecation) and ease of toilet access are the primary motivators for onsite sanitation.[28] Health and cleanliness around the house are only secondary incentives for building toilets.[28]

In addition to these household-level benefits, recent studies illustrate that building toilets and implementing fecal sludge management systems can also provide a source of revenue. Collecting human excreta, processing it and selling the value added products can provide economic opportunities and, therefore, incentives for implementing or improving sanitation systems.[29,30] HEAM (e.g., excreta/manure, wastewater, greywater) are sustainable, local and continuously available sources of nutrients for agriculture. Most of the nutrients in excreta are found in urine: 85%-90% of N, 50%-80% of P, 80%-90% of K and close to 100% of S.[31,32] However, significant amounts of these nutrients are also found in feces, which can be used as soil conditioner in agriculture, either directly or after transformation.[32,33] Besides use as a soil conditioner, fecal sludge can be processed into fuels for combustion or energy production, proteins derived from insect larvae reared on it can go into animal feed, its solid matter can go

into building materials and any remaining water can be reclaimed.[30,33]

In urban areas, this value proposition might not apply at the household scale but it can incentivize the providers of both public and private sanitation services at community and city scales. Opportunities exist, but they are highly variable from city to city, based on such factors as sludge characteristics, existing markets, local and regional industrial sectors, subsidies, and locally available materials.[30] The reuse of human excreta remains currently rare or experimental at large scale and in urban areas.[34] The spread of the necessary sanitation systems will probably require successful, sustainable examples and a paradigm shift in the perception of the waste itself - human excreta - as it is not one that most people naturally and rationally wish to deal with as a resource.[35,36]

1.2 Pathogen in HEAM

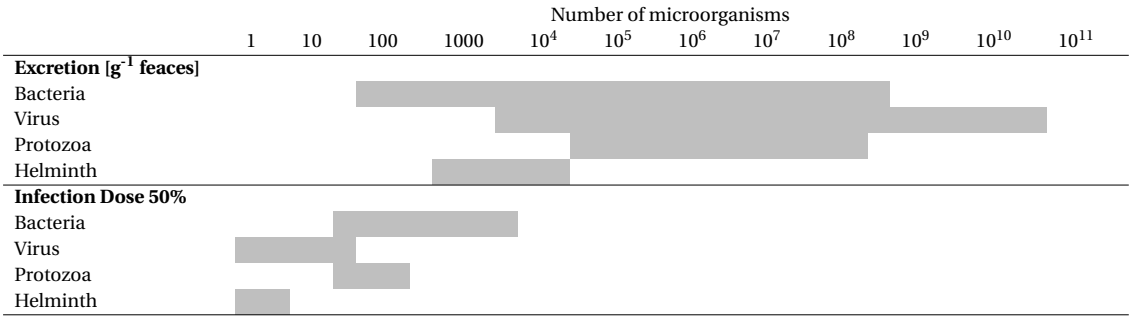
1.2.1 Pathogen type and occurence

HEAM are sources of pathogens that are mainly transmitted via the so-called fecal-oral route.[37] Among them, we find microorganisms such as bacteria, viruses, protozoa and helminthes (parasitic worms). The most common ones are listed in World Health Organization (WHO) guidelines.[38] Most of the pathogens present in sludge originate from feces.[37,38] Urine from human beings typically contains no known pathogens, but a few of them (e.g., *Schistosoma haematobium*, *Salmonella typhi*, *Salmonella paratyphi*, *Leptospira interrogans*) can be found in the urine of people infected with a corresponding disease.[37] Even in cases where sanitation systems separate urine and feces at the toilet, however, the urine collected is likely to have been cross-contaminated by the feces.[39,40] Therefore, as a baseline, human excreta should be considered to be a contaminated matrix in all sanitation systems.

Most of the fecal–oral pathogens transmitted cannot reproduce or grow outside their host. Exceptions can be found among bacteria, e.g., *V. Cholerae*,[41] which can grow in favorable environments,[42] and trematodes, such as *S. Haematobium*, that require an intermediate host for replication.[37] Fecal-oral pathogens can be released into the environment in either a highly resistant form (particularly helminth eggs, protozoa (oo)cysts and bacteria spores)[43–45] and/or in high enough numbers (particularly viruses)[46] for some to be taken up by a new host. In general, following excretion, pathogen concentrations usually decline over time depending on the environmental conditions.[37] Westrell et al.[46] compiled interesting epidemiological statistics on pathogens which showed that viruses are generally excreted in higher concentrations, yet have generally lower infection doses than other pathogens (Table 1.1).

Animal manure can also be a vector for a large number of zoonotic (i.e., infectious vertebrate diseases than can be transmitted to humans) and epizootic (i.e., epidemic among animal populations) agents. Zoonotic agents include such well-known bacteria as *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes*; other agents are the Hepatitis E virus and the protozoan *Cryptosporidium parvum*. Epizootic diseases are mainly the result of viruses,

Table 1.1: . *Range of excretion and infection doses of the four main pathogen families found in HEAM .*[46]



among which well-known ones include foot-and-mouth disease, goat/sheep pox, and avian influenza virus.[47]

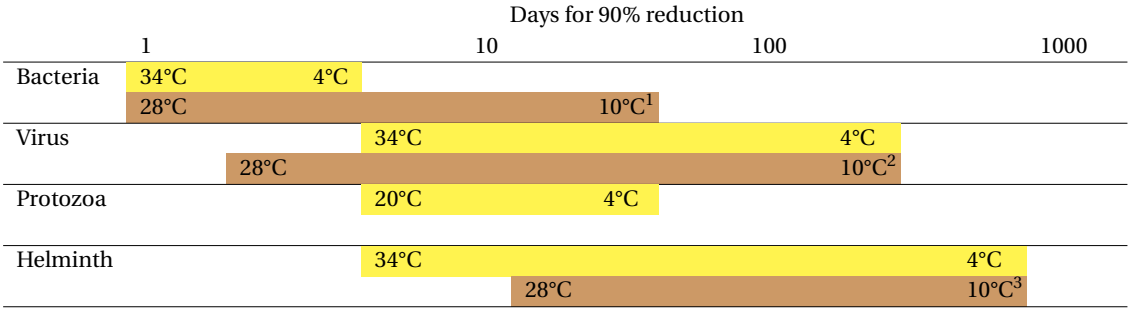
1.2.2 Pathogen containment

As described in Section 1.1, sanitation interventions aim to protect human and environmental health. In particular, interventions aim to reduce the transmission of pathogens by direct contact or ingestion or by indirect exposure through contaminated water and food.

The first step to preventing the spread of pathogens is containment. Excreta need to be contained in order to avoid uncontrolled propagation and contamination of the environment due to the most basic, simple and traditional way to pee and poo: open defecation. To do so, people can build "more or less comfortable, organized excreta containment holes", also called toilets, which can be anything from single pit latrines to flush diverting toilets through double-vault ventilated pit latrines.[48] However, building toilets is not enough. Containment systems have to be built in a way that prevents the spread of contaminants into the ground and groundwater.[49] For example, a minimum distance above the water table or from surface water must be respected,[50] and if not, efficient natural or built containment should be ensured. Achieving these standards can be a serious challenge in areas with a shallow water table and in areas with a high population density where space is not readily available. Toilets help not only to contain the excreta, but to make collection and further treatment or disposal simple.

After some time, at a rate of around 150 to 350 g day^{-1} $adult^{-1}$ (without considering volume reduction by biological activity and leakage out of the pit), toilet pits get filled with excreta.[37] They can then be filled-in and covered with soil. This is relatively safe, but it is a waste of both resources and space and, therefore, not feasible in most densely populated urban areas.[48] Thus pits and septic tanks must be emptied, usually manually or using pumps, and transported to a treatment or disposal site. The contained excreta may be partially released during these operations, and sanitation workers are especially at risk of direct or indirect ingestion of excreta and consequent exposure to pathogen contamination.[51] This is a suitable point to mention that, in reality, a non-negligible proportion of fecal sludge is not dealt with in

Table 1.2: *Range of time for 90% reduction of bacteria, virus, protozoa (C. parvum oocysts) and helminths (Ascaris eggs) in urine (yellow bar) and feces-urine mixtures (brown bar) during storage at ambient temperature, without the use of additives, i.e., potential self-sanitation.*[56,59–62] *Note that amounts of NH_3, which was shown to be a determining factor affecting inactivation (see Section 1.3.3), can vary between different stored urine and feces-urine mixtures.*



[1] Lag phase 0.5-11 days; Enterococcus more resistant than Salmonella.
[2] Lag phase 5-138 days for one of the viruses tested.
[3] Lag phase 5-338 days.

an appropriate manner; rather it is directly drained, or dumped illegally after transport, into natural surface waters. A worldwide study made in 12 cities in developing countries showed that even though household toilet coverage was high, with only 2% to 9% open defecation, the actual percentage of fecal waste safely managed (i.e., treated) varied between 2% and 92%, with an average of only 29% treated.[52] Thus, without rapid improvements in sanitation treatment coverage, on-site storage remains one of the best opportunities of reducing pathogen loads before disposal because off-site treatment units generally do not exist. Furthermore, treatment can only happen on-site if, for example, farmers used their own latrine waste as a fertilizer.[53,54] On-site storage and treatment are, therefore, a valuable means of tackling the public health problems associated with the unhygienic management of human waste.

A compilation of data available on self-sanitation of stored urine and stored feces-urine mixtures (see Table 1.2), at ambient temperatures, revealed that helminth eggs and viruses are the most resistant pathogens and that feces-urine mixtures are slower to inactivate pathogens, which is especially due to a lag phase in the inactivation process. These studies also showed that ammonia (NH_3(aq)), pH and temperature were the key parameters involved in pathogen reduction during storage. Furthermore, it was shown that pathogen reduction in feces during storage can be enhanced by using additives such as lime or urea,[55–57] or by optimizing and separating the urea hydrolysis and fecal-sludge alkalinization steps (the “pHfree Loo” toilet concept).[58]

1.2.3 Pathogen treatment

After collection and transportation, fecal sludge should be treated before final disposal or reuse in order to minimize contamination (Figure 1.3). The high nutrient and carbon content of fecal sludge need to be properly managed to lower such environmental impact as eutrophication

and oxygen depletion of natural waters.[33] Stabilizing the organic content of fecal sludge is mainly done by, for example, dewatering, aerobic/anaerobic digestion, composting or using stabilization ponds.[33,48] Most of the time, treated sludge is disposed of at a dumping site, but it can be reused as a nutrient or energy resource. In either case, an adequate level of hygiene (pathogen content) has to be reached in order to limit further pathogen release into the environment by leaching, the exposure of farmers and the contamination of agricultural and marketable products.[33]

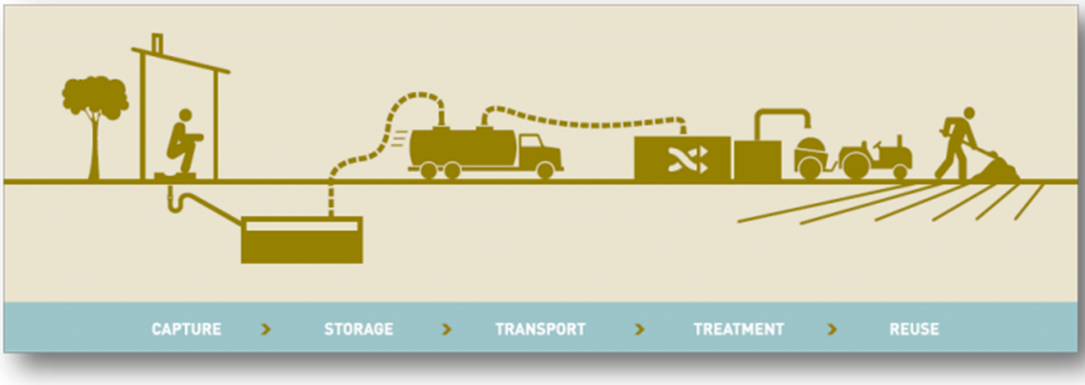


Figure 1.3: *The sanitation service chain. (source: http://sanitation.pratt.duke.edu/research/three)*

As described in Section 1.2.1, from the moment of excretion, pathogen concentrations decline over time depending on the conditions defined by ambient physicochemical and biological factors. Common parameters determining the extent of pathogen inactivation are temperature, pH and residence time.[38,63] Basic solution constituents, such as NH_3 (aq) (see Section 1.3.3) and carbonate[64,65] were also shown to enhance inactivation. Nutrient availability, competition, and grazing are common parameters determining bacterial pathogen inactivation. Microbial activity[66–69] and water content[70–72] can also influence virus survival. Sunlight is a key inactivation parameter, especially in stabilization ponds.[73,74] To a lesser extent, other substances present in waste, like detergents, were also shown to stabilize or enhance the inactivation of enteroviruses or reoviruses respectively.[75–77]

A non-exhaustive list of the options available for stabilizing and transforming human excreta includes: un-/planted drying beds, aerobic/anaerobic digestion, vermin-/co-/composting, lime/urea stabilization, solar/thermal drying, black soldier fly digestion, Latrine Dehydration and Pasteurization (LaDePa) treatment, and urine-specific treatments.[33,78,79] Even though their primary goal may not always be pathogen reduction, these processes can nevertheless lead to some removal or inactivation of pathogens; the extent of this depends on the pathogen type and the treatment conditions, i.e., the parameters involved in the inactivation step cited above.

1.3 Virus inactivation

1.3.1 Virus occurrence

Common enteric viruses found in human excreta, and associated with gastrointestinal or respiratory diseases, are reported in Table 1.3.[38,80–82] Recent metagenomic analyses of sewage sludge around the world, however, have shown larger, more complex and geographically variable human viral diversity.[83–85]

Table 1.3: *Genome type of a selection of relevant viruses found in the environment and viruses/phages used in this study. Note that all the viruses mentioned here are naked viruses.*

Genome type	ssRNA	dsRNA	ssDNA	dsDNA
Relevant human virus	Enterovirus Hepatitis A virus Hepatitis E virus Norovirus	Rotavirus		Adenovirus
Surrogate used in this study	Echovirus type 11 (EV) MS2 phage GA phage	Reovirus type 3 (ReoV)	ΦX174 phage	Human adenovirus type 2 (HAdV) T4 phage

1.3.2 Virus characteristics

Viruses are generally composed of genetic information protected by a protein coating, called the capsid, and in some cases by an additional lipid-bilayer membrane including proteins, called the envelope.[86] The genetic information may be carried in different forms (RNA/DNA, single (ss)/double stranded (ds), negative- (-)/positive-sense (+) RNA), and viruses have, therefore, a wide variety of replication strategies in their hosts. The capsid has two main functions: protecting the genome from the environment and allowing it, in the case of a non-enveloped or naked virus, to penetrate host cells (via host recognition, attachment and genome transfer). Additional proteins, essential for the infectious cycle, may be found within the capsid (e.g., DNA integrase and retro-transcriptase in a retrovirus, RNA polymerase in dsRNA viruses or some ssRNA(-) viruses, and protease in Adenoviridae). The envelope allows the virus to penetrate the host cell by membrane fusion (in contrast to a naked virus, for which penetration requires other mechanisms). The heterogeneity among viruses (e.g., genome type, capsid composition, enveloped/non-enveloped, and replication strategy) can result in dissimilar behaviors when exposed to inactivating agents. Nevertheless, general rules can be drawn when all this diversity is considered carefully.

Unlike other microorganisms, viruses are not capable of self-growth; they need a host cell to replicate their genome, produce capsid proteins, assemble the virion and, in some cases, acquire an envelope by budding through the host membrane. Although viruses do not possess any defense mechanisms against environmental stress, except the passive capsid protection of their genome, it was shown that the genome of dsDNA viruses (e.g., adenovirus) may be

repaired by host cells mechanisms in the host cell.[87]

1.3.3 Inactivation in excreta and manure

The discovery of NH_3(aq) as a viral inactivating agent. The survival of viruses in human excreta was first studied in detail (between 1975 and 1985) in the context of wastewater sludge reuse, especially during anaerobic sludge digestion. Those studies showed that virus inactivation in such heterogeneous matrices was mainly driven by temperature, a few key substances (NH_3 (aq) and detergents) and their speciation according to pH, virus characteristics and their associations with the solid matrix. For example, poliovirus infectivity was shown to decrease with time and increasing incubation temperatures, and this more rapidly in digested sludge than in raw sludge.[88,89] Notably, it was established that NH_3(aq) was the main virucidal agent of enteroviruses (polio-, coxsackie- and echovirus) (ssRNA) in digested sludge, whereas reovirus (dsRNA) was quite resistant to this compound.[90] The reasons for these differences in viral responses to similar treatment conditions were left unexplained. Furthermore, depending on the pH, ionic detergents reduced the thermal stability of reoviruses, whereas the same compounds protected enteroviruses against heat.[75–77]

Virucidal effect of NH_3(aq). The intrinsic effects of NH_3(aq) on virus inactivation were subsequently studied in more detail using buffered NH_3(aq) solutions. Ward et al. conducted an extensive study on the mechanisms of poliovirus inactivation using NH_3(aq).[91] They concluded that the RNA genome of poliovirus was the only component significantly affected by NH_3(aq) treatment. The viral RNA showed structural alteration, as observed by a decrease in the sedimentation coefficient when it was exposed to NH_3(aq) within the virion, whereas free RNA did not. In contrast, no significant capsid alteration was observed despite the loss of infectivity. No differences were observed in the viral attachment capacity, the sedimentation coefficient, the isoelectric point, or the protein pattern after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which would suggest that capsid proteins remained intact. The kinetics of virus inactivation by NH_3(aq), as well as the influence of physico-chemical factors, were also examined. Cramer et al.[92] observed that OH^- and NH_4^+ concentrations had no separate effects from those of NH_3(aq) at 20°C and in a pH range of 7–9 on coliphage f2 and poliovirus inactivation. Moreover, although they are very similar in genome type and size (both ssRNA, 7.5 and 3.5 kilo-base, respectively) poliovirus was shown to be inactivated around five times more rapidly than f2. In parallel, Burge et al.[93] isolated the effect of heat on poliovirus and phage f2 inactivation. In addition to the effect of temperature on the NH_3(aq) dissociation constant, they showed that increasing temperature increased the inactivation rate at a constant concentration of NH_3(aq). The temperature dependence of the reaction rate constant indicated biphasic behavior, although less pronounced for phage f2 than for poliovirus. A distinction could, therefore, be made between the inactivation process at low (10°C-40°C) and high (40°C-50°C) temperatures. Further calculations of thermodynamic variables (enthalpy and entropy of activation, according to Eyring's theory) suggested that inactivation occurred due to the breaking of nucleic acid chains at all temperatures for phage

f2, and at low temperatures for poliovirus. The intrinsic biocidal effect of NH_3(aq) was also demonstrated for other microorganisms, namely protozoa,[94] parasitic worms,[95] and bacteria during anaerobic digestion.[96]

Inactivation in HEAM. Virus inactivation in HEAM, such as stored urine and different types of sludge, was studied thoroughly using a wide variety of temperature, pH, and additive conditions. For the temperature (<40°C) and pH (7.0-9.5) conditions expected during the storage or mesophilic digestion of HEAM, virus concentrations were consistently shown to decrease more or less rapidly over time, depending mainly on temperature, pH, and NH_3(aq) content.[60,97] Urea and protein hydrolysis during the storage and digestion of HEAM increased both NH_3(aq) and pH.[98,99] The effect of NH_3(aq) was further confirmed by the increased virus inactivation efficiency when urea or NH_3(aq) was added to HEAM.[57,59,100,101] Heterogeneity in the sensitivity to inactivation of different viruses was also observed under these conditions.[60,90,97,101] ssRNA viruses were usually found to be more affected than viruses with other genome types.[60,90,101] As mentioned above, viral genomes were the main targets of inactivation by NH_3(aq), therefore, viral genome type seems to be a predominant factor determining inactivation. In addition to viruses, it was also demonstrated that harsh conditions in HEAM enhanced the inactivation of protozoa,[61] bacteria,[59,60,102–104] and parasitic worms.[56,59,105]

1.4 Research objectives and approach

1.4.1 Why devote an additional study to virus inactivation in HEAM?

Virus inactivation in HEAM is a complex topic because of the wide diversity of viruses and parameters potentially involved in the process. As described in the previous section and in other literature, there is a lot of information on virus inactivation available, covering a range of viruses and sludge treatment processes. However, due to the difficulties in monitoring experimental parameters, studies that systematically control parameters are rare. Furthermore, systematic studies are often conducted in complex matrices within which differentiating the effects of each parameter involved in the inactivation is difficult.[60,101] The complete characterization of the matrices used in these studies would seem to be strategic, however it is often overlooked. Since Ward et al.[90] discovered that NH_3(aq) was a critical parameter in determining the inactivation of ssRNA viruses in sludge, few mechanistic[91] or kinetics[92,93] studies have been conducted on this topic, as described in Section 1.3.3. Furthermore, those studies focused on NH_3(aq) and ssRNA viruses, without questioning why other viruses might be less sensitive. It thus seems necessary to take a step back from evaluating virus inactivation in the field, and to focus on a search for more fundamental knowledge. As Pecson showed with his focus on Ascaris egg treatment in sludge,[106] drawing out simple rules that guarantee the inactivation of pathogens will ultimately help us to interpret the overall complexity of pathogen inactivation in real matrices, and enable us to focus our attention on which crucial parameters we must control in the field. The primary objectives of the present study are to pinpoint some of those rules for viruses and to shed light on the “black box” processes that

govern virus inactivation.

1.4.2 Research questions and strategies

We focus on inactivation during the storage and mesophilic digestion of HEAM. Thus, our study mainly concentrates on the temperatures <40°C and mildly alkaline pH conditions (7.0–9.5) which occur naturally during those steps. This thesis is divided into three research chapters, all directly linked to each other. The chapters aim to answer the following research questions:

Chapter 2. Why are ssRNA viruses sensitive to NH_3(aq)? What are the relationships between temperature, pH and NH_3(aq) during virus inactivation? Is NH_3(aq) the only virucidal agent, or are there other compounds with similar properties to NH_3(aq) that could enhance inactivation? Is it possible to predict the inactivation of a model ssRNA virus in simple solutions?

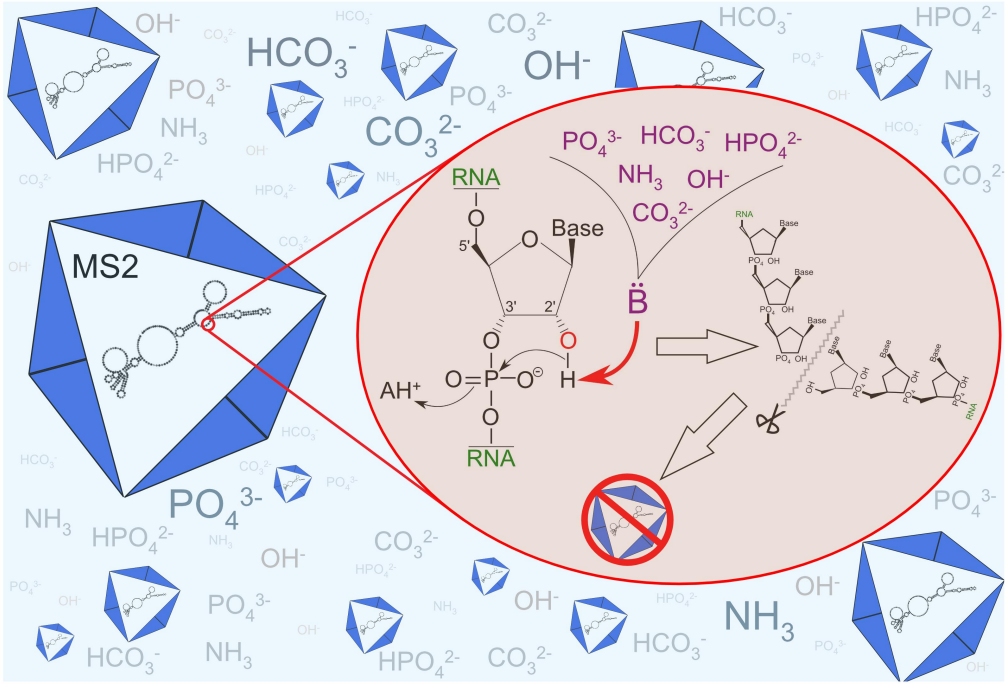
Chapter 3. What is the relationship between virus characteristics, such as genome type, and viral inactivation behavior? What happens to the heterogeneity of virus inactivation if we push the system beyond the temperature and pH limits of mesophilic systems? How can we predict these different inactivation behaviors? Can we predict inactivation for all type of viruses, as in Chapter 2, or do we need to rely on worst case indicators?

Chapter 4. Are the observations made in Chapters 2 and 3 still valid inside real matrices, such as stored urine and sludge? Can we still predict the inactivation of a model ssRNA virus in stored urine or sludge? What additional inactivating agents are present in real matrices?

This thesis used the following strategies to address these research questions:

	Virus tested	*Type of solution*	*Critical parameters evaluated*
Chapter 2	phage MS2	Synthetic	T, pH, base content
Chapter 3	phage MS2, GA, T4, ΦX174 virus HAdV, Reo, EV	Synthetic	T, pH, NH_3
Chapter 4	phage MS2, T4, ΦX174 virus HAdV	Stored urine, sludge	T, pH, solution constituents, microbial activity

2 Inactivation kinetics and mechanisms of the ssRNA virus MS2 by NH_3(aq)



2.1 Introduction

Uncharged aqueous ammonia (NH_3(aq)) is known to have sanitizing properties against pathogenic microorganisms present in human and animal excreta (HEAM).[94–96] NH_3(aq) is released by urea and protein hydrolysis during storage and digestion of HEAM.[99,107] As a consequence, high amounts of total ammonia (NH_4^+ + NH_3(aq); pK_a (25 °C) = 9.25) are present in stored urine, stored fecal sludge and anaerobically digested sludge. NH_3(aq) can thus be considered a natural sanitizing agent produced *in situ* that can be exploited to decontaminate HEAM.

Viruses constitute an important group of pathogens present in HEAM, yet the biocidal efficiency of NH_3(aq) toward viruses is poorly understood. Experiments performed under controlled conditions demonstrated that virus inactivation is directly related to the concentration of NH_3(aq) over a pH range of 7-9.[91–93] Similarly, recent studies investigating virus inactivation kinetics in complex matrices reported an increase in inactivation with increasing NH_3(aq) concentration.[60,97,100,101,108–112] However, these studies did not consider additional inactivating parameters present in the experimental systems, and hence the findings cannot be generalized across different matrices.

The mechanisms associated with virus inactivation by NH_3(aq) were investigated by Ward et al., who determined that for RNA viruses the main target of NH_3(aq) is the genome.[91] Further evidence of genome involvement can be obtained by comparing the outcomes of different studies on the kinetics of virus inactivation by NH_3(aq): in complex matrices single-stranded (ss)RNA viruses were consistently inactivated by NH_3(aq),[60,90,92,93,101] whereas the double-stranded (ds)RNA reovirus and rhesus rotavirus as well as the dsDNA salmonella phage 28B were less affected.[60,90,101] Among ssRNA viruses, differences in the susceptibility to NH_3(aq) were also observed, though this variability was small compared to the differences between ssRNA viruses and those of other genome types.[101] Combined, these data suggest that the heterogeneity among viruses in their susceptibility toward NH_3(aq) is driven by the genome type, thus further implicating the genome as the target of NH_3(aq).

Although previous work provides a qualitative understanding of the virucidal effects of NH_3(aq), the extent of virus inactivation in HEAM can currently not be predicted quantitatively. In the present study we provide the necessary knowledge to allow such predictions for MS2, an ssRNA phage that is commonly used as a surrogate for human viruses. We focused on an ssRNA virus because it represents the genome type of most human pathogenic viruses, and because ssRNA viruses were shown to be the most sensitive toward NH_3(aq). Experiments were conducted under controlled conditions relevant to HEAM treatment, that is, under mildly alkaline pH conditions (pH 7-9.5), common HEAM storage temperatures (4-35 °C) and range of ammonia activities typically observed in stored urine and anaerobically digested sludge (0-200 mmol L^{-1}).[60,113] First, the kinetics of virus inactivation by NH_3(aq) were studied as a function of pH, temperature, and ion activity. Second, we elucidated the mechanisms of inactivation by quantifying the degradation of the different virus components and the associated loss in virus

functions. Finally, the observed virus inactivation was compared to that predicted based on the kinetic and mechanistic insights gained, and general conclusions on virus inactivation by NH_3(aq) were drawn.

2.2 Materials and Methods

Phages and bacteria. Coliphage MS2 (DSMZ 13767) and its host *Escherichia coli* (DSMZ 5695) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). MS2 was propagated as described previously,[114] and infectivity was assessed by enumeration of plaque forming units (PFU) using the double agar layer method.[115]

Chemicals. Buffer solutions were made with ammonium chloride (NH_4Cl; Acros), Imidazole (Acros), potassium chloride (KCl; Acros), sodium carbonate (Na_2CO_3; Fluka), sodium chloride (NaCl; Acros), and sodium phosphate (NaH_2PO_4; Acros).

Experimental setup. Two types of kinetic experiments were performed: in the first, the effect of NH_3(aq) in mixed buffered solutions was assessed; in the second, the effect of other solution constituents, in particular other bases, was determined. Experiments relating to the effect of NH_3(aq) were conducted in controlled solutions at different ranges of pH (7- 9.5), temperature (T; 4-35 °C), and NH_3(aq) activities ({NH_3(aq)}; 0-160 mmol L^{-1}). Experiments were performed in ammonium carbonate buffer (AmCa) produced from Na_2CO_3 and NH_4Cl. NH_3(aq)-free controls were conducted in phosphate carbonate (PCa; Na_2CO_3 and NaH_2PO_4) or potassium carbonate buffer (KCa; Na_2CO_3 and KCl). For all {NH_3(aq)}, T and pH conditions tested, the Na_2CO_3 concentration was kept constant at 50 mmol L^{-1}. The exact buffer composition for each experiment can be found in the Appendix A, Table A.1. To determine the effect of other bases (phosphate, (bi)carbonate, imidazole), experiments were conducted in solutions containing only the base under consideration. These experiments were carried out at a constant temperature (35 °C) and pH, and at different base activities. The experimental details can be found in Appendix A, Table A.2. Prior to all experiments, the composition of the buffer was estimated with PHREEQC (version 2.18.00) in order to approximate the desired experimental conditions to be tested. Buffer solutions were freshly prepared, the pH was adjusted by the addition of HCl or NaOH (Acros) and the mixture was stored tightly sealed for 1 h to dissolve any remaining salts. Sterilized glass serum flasks (Infochroma) were then filled with 114 mL of the buffer solution, closed with a sterilized airtight cap, and stored at the targeted temperature for at least 1 day. To initiate the inactivation experiment, one milliliter of an MS2 solution containing of 10^{10}-10^{11} PFU mL^{-1} in virus dilution buffer (VDB; 5 mmol L^{-1} NaH_2PO_4, 10 mmol L^{-1} NaCl, pH adjusted to 7.5 with NaOH) was added to the serum flasks containing 100 mL buffer to achieve a final concentration of 10^8-10^9 PFU mL^{-1} in the flask. Samples were periodically taken with a sterile syringe, were serially diluted in VDB, and were stored at 4 °C until enumeration (within a few hours).

Activity calculations. At the end of each experiment the pH was measured at the experimental

temperature (780 pH Meter with primatrode with NTC no. 6.0228.010, Metrohm, Herisau, Switzerland). For the experiments involving NH_3(aq) the total ammonia concentration was determined by ion chromatography (ICS-3000A, IonPacCS16 column) with electrical conductivity detection (Dionex, Switzerland). For every experimental condition the pH was reported as the average pH measured in duplicate flasks. Total ammonia was measured four times per flask. {NH_3(aq)} was calculated based on the average of the four measurements, the average measured pH and the added Cl^-, CO_3^{2-}, and Na^+ concentrations using PHREEQC and a database using the Pitzer approach for calculating the ion activity. Note that the concentration expressed in mol kg^{-1} in PHREEQC were considered equivalent to mol L^{-1}. The reported data range reflects the 95% confidence intervals. For the experiments involving other bases the base activity was calculated from the added amount of base, the average measured pH and the added Na^+ or Cl^- concentrations using PHREEQC and the database mentioned above. In the case of imidazole a modified wateq4f database was used, which uses the Debye-Hückel approach to determine ion activity.[116]

Kinetic data analysis. Each experimental condition was tested in duplicate. Phage titers in each flask were determined in duplicate or triplicate. Inactivation kinetics were determined by least-square fit of a first-order model to the data according to the following equation:

$$ln\frac{C}{C_0} = -k_{obs}t \tag{2.1}$$

where C_0 and C [PFU mL^{-1}] are the concentrations of MS2 at time 0 and t and k_{obs} is the observed first-order inactivation rate constant [day^{-1}]. The data of all replicates were pooled and the 95% confidence interval of k_{obs} was calculated from the standard error of the slope of the pooled data. The second-order rate constant associated with an inactivating species j (k_j; [day^{-1} L mol^{-1}]) was determined by the best fit of a linear model according to the following relationship:

$$k_{obs} = k_j\{j\} + k_{background} \tag{2.2}$$

where j is the activity of the inactivating species j under consideration [mol L^{-1}] and $k_{background}$ is the first-order rate constant of the background solution [day^{-1}] in absence of j. In the case of CO_3^{2-} and HCO_3^-, the respective k_j could not be experimentally determined individually. Therefore, each k_j was determined by a linear least-squares fit based on a two parameter model ($k_{obs} = k_{CO_3}*\{CO_3\} + k_{HCO_3}*\{HCO_3\} + k_{background}$) with data obtained at pH 8.5 and 9.5. k_j for NH_3(aq) (k_{NH_3}) for different pH at 20 and 35 °C were compared by means of an ANCOVA analysis.

Binding assay. The effect of NH_3(aq) on MS2's ability to bind to the host cell was evaluated after different times of exposure to AmCa buffer ({NH_3(aq)} = 40 mmol L^{-1}, pH 9.0 and 9.5, 35 °C) as described previously.[117] In brief, MS2 samples were incubated with Escherichia coli for 90 min at 4 °C which allowed the viruses to attach to the bacterial host without injecting or replicating their genomes. The samples were then centrifuged and the bacterial pellet

was collected, rinsed to remove unbound viruses, resuspended in VDB, and finally the viral RNA was extracted and enumerated by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Genome integrity assay. Genome integrity was measured for the intact virus (encapsulated genome; i.e., extraction of the genome after exposure of the intact virus to $NH_3(aq)$) in AmCa ($\{NH_3(aq)\}$ = 40 mmol L^{-1}) at pH 8.0/20 °C, pH 9.0/35 °C and pH 9.5/35 °C. The degradation of the encapsulated genome in AmCa ($\{NH_3(aq)\}$ = 40 mmol L^{-1}) at pH 8.0/20 °C was compared to that of the naked genome (extraction of the genome prior to its $NH_3(aq)$ exposure) under experimental conditions. The starting genome concentration was 10^{10} genome copies (gc) mL^{-1}, which corresponds to the genome content of a solution containing 10^9 PFU mL^{-1} infective MS2. Both encapsulated and naked genome samples were collected periodically, were immediately extracted as described previously,[114] and were stored at -20 °C until analysis by RT-qPCR. Three different genome segments of approximately 300 nucleotides (nt) each were analyzed using three primer pairs described previously (primer set 3, 6, and 12 according to Pecson et al.).[114] For the encapsulated samples, infectivity was measured in parallel. Overall genome degradation was calculated by extrapolating the damage measured in the three genome segments to that across the whole genome according to the following expression:[118]

$$\frac{N_t}{N_0} = \left(\prod_i \frac{n_{it}}{n_{i0}}\right)^{\frac{genome}{\text{total length of three genome segments}}} \tag{2.3}$$

where N_t/N_0 is the proportion of the entire genome that remains intact at time t, n_{it}/n_{i0} is the measured proportion of intact genome segment i, and i is the genome segment tested at time t (i = 3, 6, or 12). Hereby the term "intact genome segment" refers to a segment that the RT-qPCR process could amplify. To determine if $NH_3(aq)$ affected all genome regions at equal rates, the degradation kinetics of each segment i were analyzed separately. Degradation was assessed by calculating the degradation rate constant $k_{obs,segment}$ as a function of inactivation [day^{-1}]:

$$ln\frac{n_{it}}{n_{i0}} = -k_{obs,segment}\,t \tag{2.4}$$

Because the degradation rate may vary with the length of the genome segment, each $k_{obs,segment}$ was normalized ($k_{norm,segment}$; [day^{-1}nucleotide^{-1}]) by its length, which corresponded to 303, 289, and 245 nucleotides for genome segments 3, 6, and 12, respectively.[114] Finally, to compare genome damage under different experimental conditions, $k^*_{norm,\,segment}$ [ln(C/C0)$^{-1}$nucleotide^{-1}] was introduced, which denotes the degradation rate constant as a function of inactivation. The influence of the experimental variables on $k^*_{norm,segment}$ was assessed by two-factor ANOVA.

Genome electrophoresis. Electrophoresis of MS2 genomes was performed to observe the genome cleavage pattern during the course of an inactivation experiment. Inactivation experiments were performed at 20 °C in VDB, PCa (pH 9.0) and AmCa (pH 9.0, $\{NH_3(aq)\}$ = 40

mmol L^{-1}), and samples were periodically collected and subjected to electrophoresis. Electrophoresis was carried out with Agilent RNA 6000 pico kit and 2100 Bioanalyzer (Agilent Technologies Inc.). To accommodate the high detection limit of the electrophoresis instrument, experiments were conducted in 10 mL serum flasks (Infochroma) with an initial MS2 concentration of 10^{11} PFU mL^{-1}. Five hundred microliters were sampled, diluted 10-fold, and were extracted as described previously.[114] Samples were then prepared and analyzed according to the manufacturer instructions.

2.3 Results and Discussions

Inactivation kinetics: influence of pH and temperature on MS2 inactivation by NH_3(aq).

Influence of NH_3(aq). The MS2 infectivity in all AmCa solutions was reduced by at least one log_{10} unit over 5 days. First-order inactivation kinetics were observed for all temperatures (4 to 35 °C), pH values (7.5-9.5) and {NH_3(aq)} (10 to 160 mmol L^{-1}) (Figure 2.1 and Appendix A, Table A.1).

Furthermore, first-order rate constants showed a linear relationship with {NH_3(aq)} (Figure 2.2a), indicating that MS2 inactivation is first-order in {NH_3(aq)}. This is consistent with previous reports by Cramer et al.,[92] who also determined a linear relationship for {NH_3(aq)} between 0.5 and 176 mmol L^{-1}.

Influence of pH. The pH indirectly affects NH_3(aq)-mediated inactivation kinetics by determining NH_3(aq) speciation. At a given total ammonia concentration, an increase in pH leads to an increase in {NH_3(aq)}, and hence a proportional increase in k_{obs} (eq. 2.2). This behavior was observed for the entire pH range considered at a temperature of 35 °C (note the equal slopes in Figure 2.2a). Consequently, the second-order rate constant for inactivation by NH_3(aq) (k_{NH_3}) determined at each of these pH values were equal at 35 °C ($F(4,16) = 0.624$, $p = 0.652$). Similar results were obtained at 20 °C (Figure 2.3). This supports the notion that in a pH range of 7.5-9.5, NH_3(aq) is an important reactive entity in these solutions, and that pH does not influence the virus' susceptibility to NH_3(aq) (no change in k_{NH_3}). It furthermore confirms the observations by Cramer et al. who reported no influence of the pH on f2 phage inactivation by NH_3(aq) from pH 7.0 to 9.0 at 20 °C.[92]

At pH values of 9.5 an additional inactivating effect was observed (Figure 2.2a and Figure 2.3a): at any given {NH_3(aq)} k_{obs} was higher at pH 9.5 than at lower pH values, and this difference stayed constant with increasing NH_3(aq). This effect could be accounted for by introducing a $k_{background}$ (eq. 2.2), which corresponds to the k_{obs} at {NH_3(aq)} = 0 mmol L^{-1}. A similar effect was observed at pH 9.0, though it was only evident at low {NH_3(aq)}. This indicates that the pH-induced increase in virus inactivation was due to the increased activity of other bases in the background solutions, such as hydroxide, (bi-) carbonate or phosphate. The contribution of these solution constituents to MS2 inactivation is elucidated in further detail below.

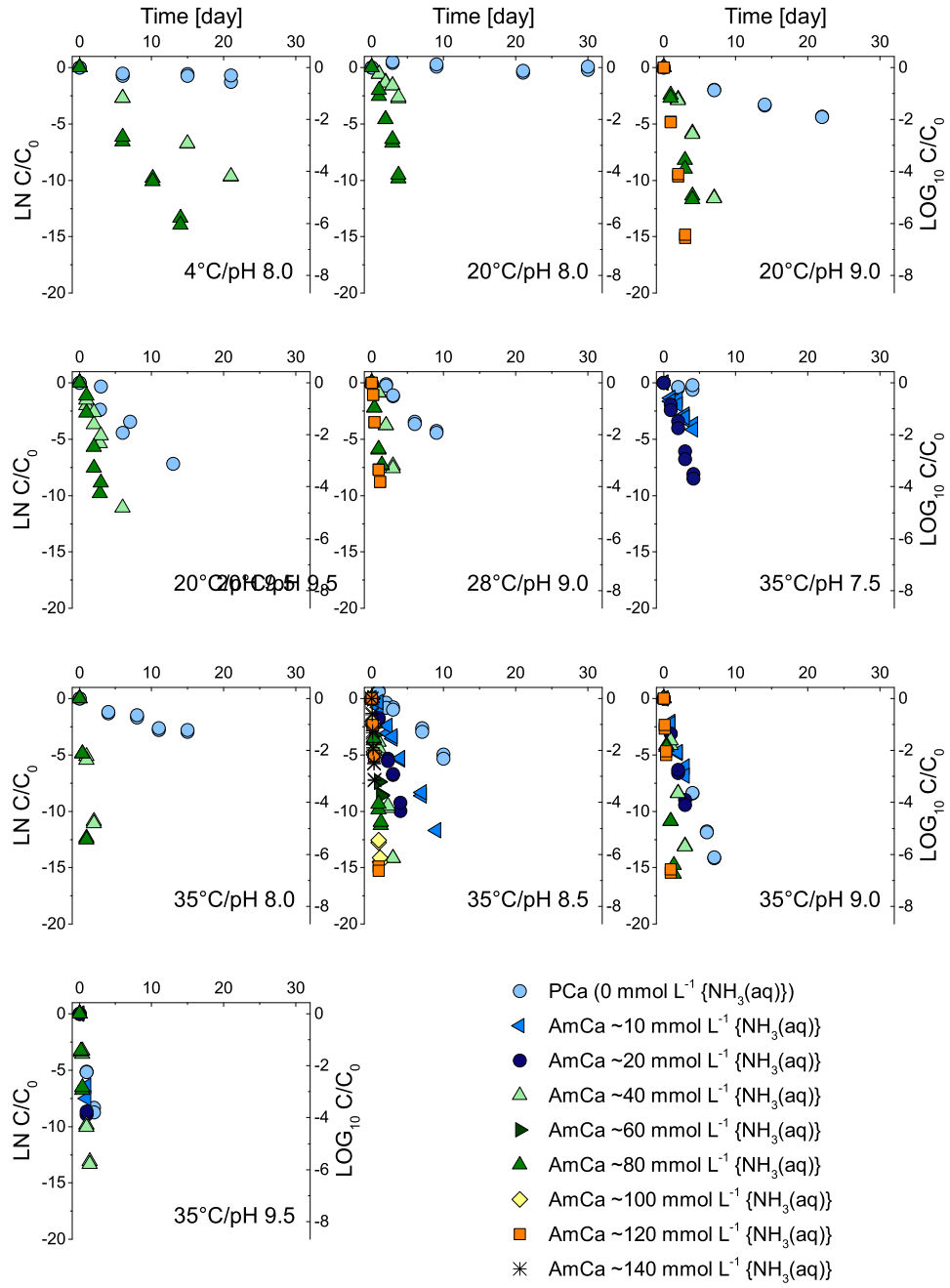


Figure 2.1: *Kinetics of MS2 inactivation in PCa and AmCa solutions. Intended $NH_3(aq)$ are shown in the legend and measured $NH_3(aq)$ can be found in Appendix A, Table A.1.*

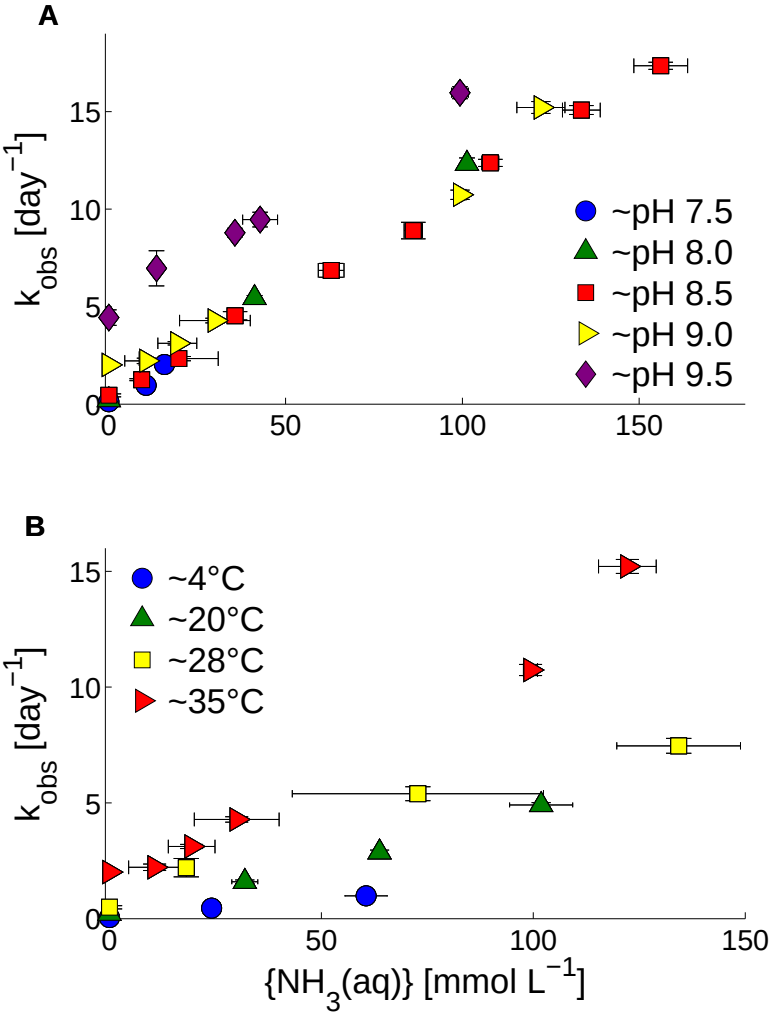


Figure 2.2: *First-order rate constants (k_{obs}) of MS2 inactivation in controlled solutions. (A) Dependence of k_{obs} on pH and {NH_3(aq)} at 35 °C. (B) Dependence of k_{obs} on temperature and {NH_3(aq)} at pH 9.0. Vertical error bars depict 95% confidence interval associated with k_{obs}. Horizontal error bars represent 95% confidence intervals determined based on four replicate {NH_3(aq)} measurements.*

Influence of temperature. In addition to pH, k_{obs} also depended on temperature. Temperature can act on all three parameters constituting k_{obs}: the NH_3(aq) speciation, the second order rate constant k_{NH_3} and $k_{background}$ (eq. 2.2). The influence of temperature on speciation is substantial; between 4 and 35 °C, {NH_3(aq)} increases by a factor of 5 at a given buffer composition and total ammonia concentration.

The effect of temperature on k_{NH_3} is illustrated in Figure 2.2b and Figure 2.3b: an increase in temperature led to a stronger dependence of k_{obs} on {NH_3(aq)}, indicating that k_{NH_3} increased with temperature. The temperature dependence of k_{NH_3} can be quantified with the Arrhenius relationship, which showed linear dependence of ln k_{NH_3} on 1/T (Figure 2.3c). This

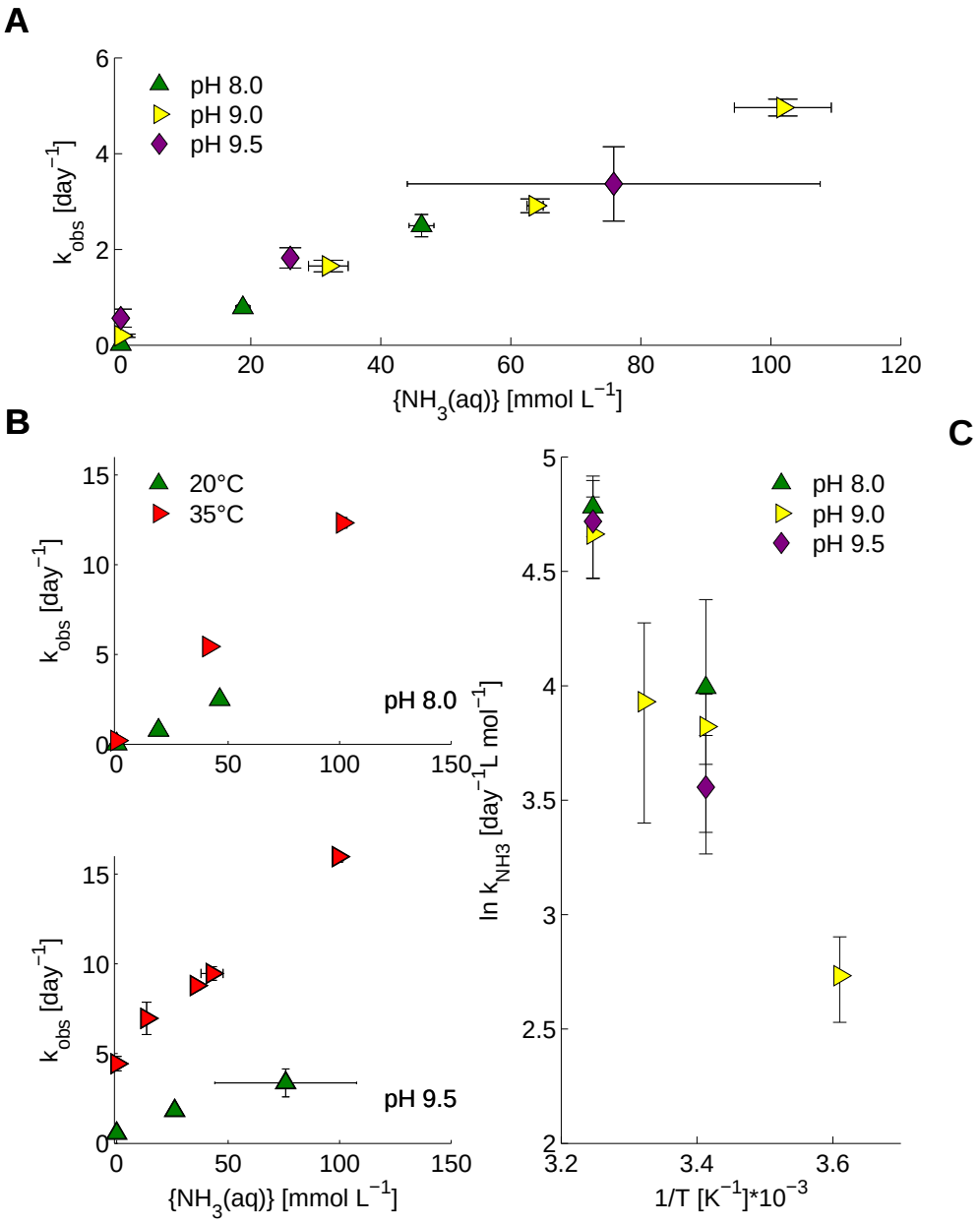


Figure 2.3: *MS2 inactivation first-order rate constant in controlled solutions.(A) Effect of pH and {NH_3(aq)} on inactivation at 20°C. The slopes at the 3 different pH are not significantly different (F(1,5)=2.468, p=0.177, by however removing the last data of pH 9.5 which showed large error).(B) Effect of temperature and {NH_3(aq)} on inactivation at pH 8.0 and 9.5 .For (A) and (B), vertical error bars depict 95% confidence interval associated with k_{obs}. Horizontal error bars represent 95% confidence intervals determined based on four replicate {NH_3(aq)} measurements. (C) Arrhenius plot for the k_j at the different pH. Vertical error bars depict 95% confidence intervals of k_j.*

suggests that the same temperature-dependent process is involved in virus inactivation over the temperature range of our experiments (4-35 °C). Within this range, a doubling of k_{NH_3} can be expected for every ~11 °C increase in T. At temperatures above 40 °C, Burge et al. observed a biphasic Arrhenius plot for f2 phages and poliovirus in NH_3(aq) containing solutions (pH 8.0, [NH_3(aq)] = 300 mg L^{-1}), indicating that other or additional processes start to dominate virus inactivation at higher temperatures.[93]

Finally, temperature also affected $k_{background}$, albeit in a pH-dependent manner. As can be seen in Figure 2.2b, $k_{background}$ increased with rising temperatures at pH 9. A more pronounced effect of temperature on $k_{background}$ was observed at pH 9.5 (Figure 2.3b). A likely explanation is that the rate-enhancing effect of non-NH_3(aq) bases on MS2 inactivation increased with temperature.

In summary, it can thus be stated that MS2 inactivation by NH_3(aq) is affected by both pH and temperature, though their influence on k_{obs} differs: pH and temperature both act by influencing the NH_3(aq) speciation, whereas temperature additionally affects k_{NH_3}. Over the experimental range considered, no synergistic effects of NH_3(aq), pH and temperature were evident. Furthermore, NH_3(aq) was shown to be the main inactivating compound in the experimental system used in this study except at pH 9.5, where the inactivating effect of the background solution was also prominent. When considering the mechanisms of inactivation in the following section, we therefore focus on the effect of NH_3(aq) and increasing pH.

Effect of NH_3(aq) and increasing pH on virus function and components.

Effect of NH_3(aq) and increasing pH on MS2 proteins. Virus inactivation is caused by a modification of the viral genome or proteins, which ultimately cause the virus to lose its ability to perform vital functions. The binding of the virus to the host cell is the first step for successful infection. This is followed by the transfer of the viral genome into the host. Both these functions are mediated by viral proteins, and hence a loss in host binding or genome internalization can be attributed to a modification of proteinaceous virus components. Here, we used host binding as a proxy to assess the extent of protein damage by NH_3(aq).

NH_3(aq)-mediated inactivation did not exert much effect on the integrity of proteins involved in host binding: only about 5% of total inactivation could be accounted for by a loss in this viral function (Figure 2.4). This implies that NH_3(aq) only leads to a low extent of protein modification.

At higher pH, an NH_3(aq)-independent contribution to k_{obs} was observed (Figure 2.2a) that was accompanied by a greater loss in host biding. At pH 9.5, binding loss accounted for approximately 20% of inactivation (Figure 2.4). Given that all host binding experiments were conducted at the same {NH_3(aq)}, this increase in binding loss can be attributed to an effect of non-NH_3(aq) bases on viral proteins. The finding that other bases prevalent at high pH, but not NH_3(aq), influence binding is consistent with observations by Ward et al., who showed that the presence of NH_3(aq) did not affect the binding capacity of poliovirus at pH

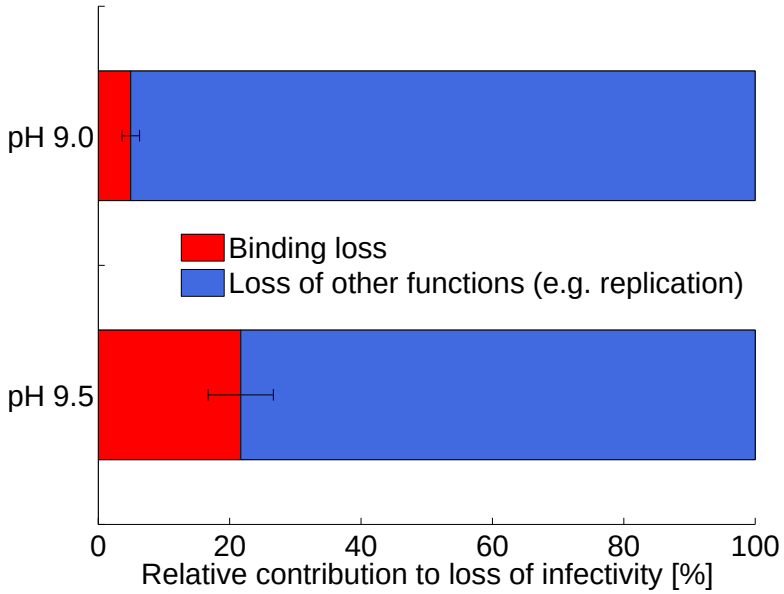


Figure 2.4: *Contribution of binding loss to overall infectivity loss of MS2 in AmCa ({$NH_3(aq)$} = 40 mmol L^{-1}) at 35°C. Error bars depict 95% confidence intervals of duplicated assay. A heat-inactivated (72°C) MS2 sample, which has previously been shown to completely inhibit phage binding to the host served as the positive control and show 100% binding loss (data not shown).*

9.5.[91] A deteriorating effect of high pH on viral proteins has also been reported by others and was attributed to an increase in the rate of peptide bond hydrolysis at pH 10 and above.[119] For example, the poliovirus capsid proteins and nucleocapsid of white spot syndrome virus were found to be affected starting at a pH of around 10.[120,121] However, for environmentally relevant temperatures (4-35 °C) and pH values of 9.5 and below, our results indicate that MS2 protein integrity is conserved and that the genome is therefore likely the main viral constituent involved in the loss of infectivity.

Effect of $NH_3(aq)$ and increasing pH on the MS2 genome. Genome integrity was assessed by means of electrophoresis (Figure 2.5a) and RT-qPCR (Figure 2.5b and Figure 2.6). The first method provided a qualitative insight into the integrity of the whole genome, whereas the latter enabled a quantitative measure of the degradation of selected segments of the genome. A decrease of the MS2 genome peak (genome size 3569 nucleotides) in both PCa and AmCa solutions was observed by electrophoresis at 20 °C and pH 9, though the decrease in AmCa was more pronounced (Figure 2.5a). This effect was evident even though the initial peak at t = 0 days in AmCa was consistently reduced compared to that of PCa or VDB. The reason for this reduction in initial peak size is not known. In contrast, the MS2 genome remained intact at neutral pH under the same temperature conditions (VDB, pH 7.5; Figure 2.5a). The observed genome degradation in the absence of NH_3(aq) (in PCa buffer) at pH 9 indicates that the high pH or other compounds present in PCa contributed to the cleavage of MS2 genome.

The kinetics of genome degradation were measured for three different segments of the MS2

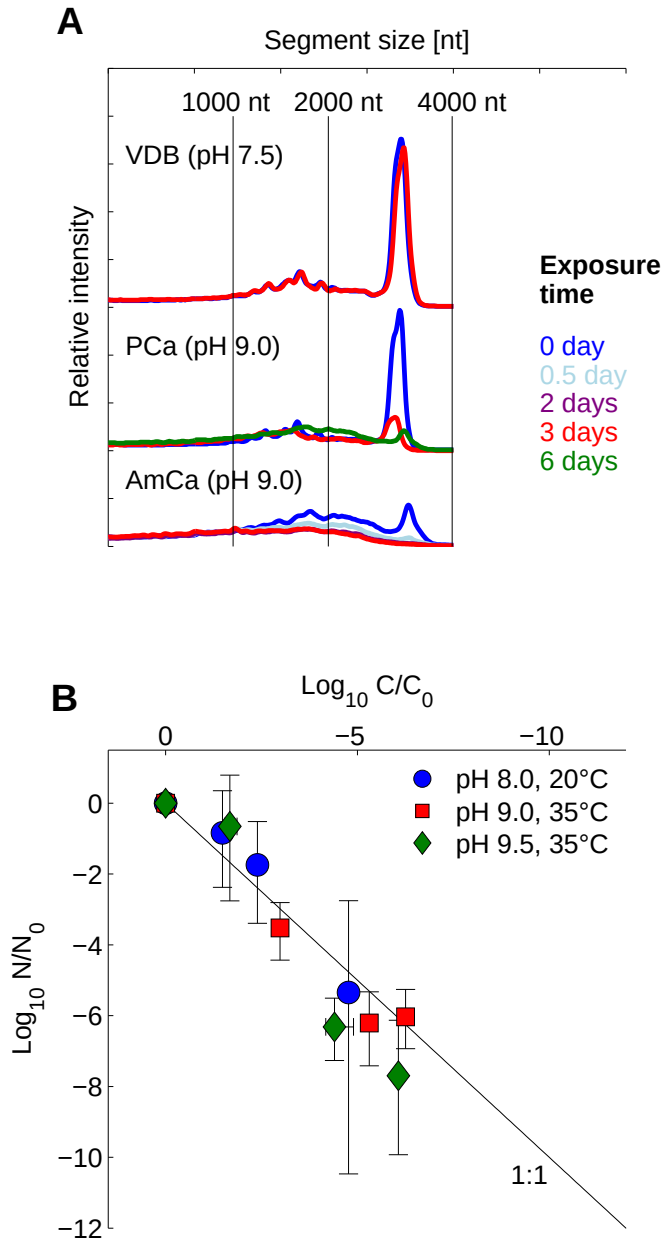


Figure 2.5: *MS2 genome integrity: (A) electrophoresis measurement of MS2 genome after different times of exposure to VDB, PCa and AmCa ({NH_3(aq)} = 40 mmol L^{-1}) at 20 °C; (B) Comparison of MS2 infectivity loss (C/C_0) to genome degradation (N/N_0) in AmCa ({NH_3(aq)} = 40 mmol L^{-1}) at pH 8.0/20 °C, pH 9.0/35 °C and pH 9.5/35 °C. Vertical error bars depict standard deviation of triplicate measurements. The straight line depicts the 1:1 ratio.*

genome by RT-qPCR for three conditions: pH 8.0/20 °C, pH 9.0/35 °C, and pH 9.5/35 °C in AmCa ({NH_3(aq)} = 40 mmol L^{-1}) (Figure 2.6a). Neither segment type ($F(2,4) = 1.621$, $p = 0.305$) nor experimental conditions ($F(2,4) = 1.848$, $p = 0.27$) had a significant effect on $k^*_{norm,segment}$, indicating that NH_3(aq)-mediated genome degradation was homogeneous across the genome.

This was further supported by the electrophoresis data, which did not show any preferential pattern attributable to a specific, consistent cleavage product (Figure 2.5a).

The kinetics of degradation of the whole genome were then extrapolated from these three segments (eq. 2.3) and were compared to the inactivation kinetics. As can be seen in Figure 2.5b, a 1:1 relationship between genome degradation and infectivity loss was found. In other words, every unit of genome loss was accompanied by a unit of infectivity loss for all experimental conditions tested. Recalling that protein integrity was retained under our experimental conditions, we can thus conclude MS2 was inactivated by single-hit genome degradation. These findings are consistent with previous reports that identified the genome as the main target of NH_3(aq).[91] They are furthermore supported by the thermodynamic properties associated with the reaction of NH_3(aq) with ssRNA viruses: within a temperature range from 10 to 40 °C, a low enthalpy and a negative entropy of inactivation were determined, which suggests that inactivation involved the breakage of ssRNA (protein and DNA breakage are associated with higher enthalpies) and the formation of a less stable structure through hydrolytic cleavage of the phosphodiester nucleotide linkage, respectively.[93]

Mechanism of genome degradation. Genome degradation can be promoted by enzymes or by harsh chemical conditions. Enzymatic activity has been put forward to explain the NH_3(aq)-mediated degradation of the poliovirus genome. Ward et al.[91] compared the effect of NH_3(aq) on naked and encapsulated poliovirus RNA and found that viral RNA within the capsid degraded, while naked RNA stayed intact. Some authors have hypothesized that the presence of nonstructural proteins with endonuclease activities within poliovirus capsids may explain these observations.[122,123] The endonuclease activity was shown to be enhanced by the presence of monovalent cations (e.g., NH_4^+, Cs^+, and K^+),[122–124] and may thus cause degradation of the encapsulated genome in the presence of NH_4^+. In the case of MS2, however, a comparison between naked and encapsulated genome segments did not reveal any differences in genome degradation rates (Figure 2.6b). Furthermore, the presence of endonucleases within MS2 has never been reported. Finally, a comparison of MS2 inactivation in PCa, AmCa, and KCa buffer with similar NH_4^+ and K^+ activity showed that inactivation in KCa was comparable to that in PCa (data not shown). Therefore, endonuclease activity can be excluded as the cause of genome degradation in MS2.

An alternative to endonuclease-driven genome cleavage of ssRNA is the nonenzymatic hydrolysis of the RNA sugar-phosphate backbone. The RNA ribose contains a hydroxyl group at the $2'$ position, which confers substantial instability to RNA compared to DNA deoxyribose. The presence of the nucleophilic oxygen of the $2'$-hydroxyl group close to the phosphodiester enables RNA cleavage by base-catalyzed transesterification (Figure 2.7).[125–129]

The composition of the bulk solution, in particular the concentration of bases, is a key parameter determining RNA transesterification kinetics. Base strength was shown to be positively correlated with RNA transesterification rates,[130,131] and hence base-catalyzed RNA transesterification can be considered a general base catalysis reaction. This implies that the

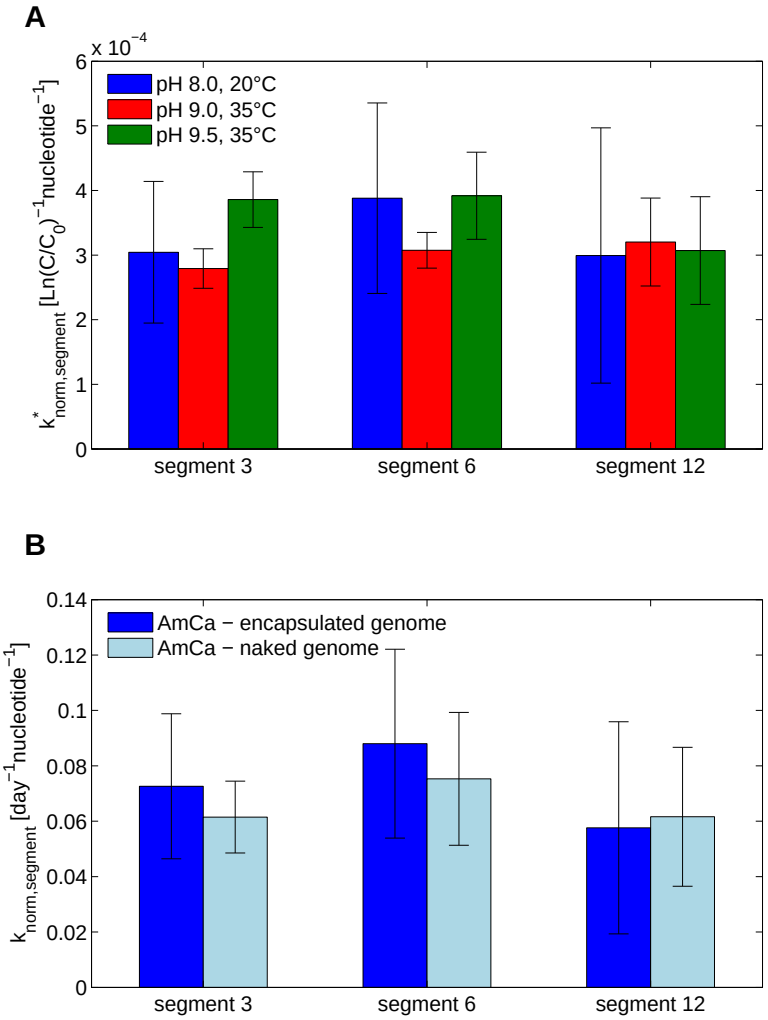


Figure 2.6: *(A) Degradation rate constant as a function of inactivation ($k^*_{norm,segment}$) of three different genome segments of MS2 upon inactivation in AmCa (40 mmol L^{-1} {NH_3(aq)}) at pH 8.0/20°C, pH 9.0/35°C and pH 9.5/35°C. (B) Normalized first-order decay rate ($k_{norm,segment}$) of 3 different genome segments of MS2 after inactivation in AmCa (40 mmol L^{-1} {NH_3(aq)}) at pH 8.0 and 20°C. Genomes were exposed within intact virus (encapsulated) or after extraction (naked). Vertical error bars depict 95% confidence intervals of $k_{norm,segment}$ and $k^*_{norm,segment}$.*

transesterification rate is dependent on the pH as well as on the concentration of the different bases in solution, and that the base-promoted deprotonation of the 2′-hydroxyl group of the ribose by the base is the rate limiting step.[129,132] Considering our findings that the inactivation of MS2 in AmCa is proportional to the activity of the base NH_3(aq) and that the MS2 genome is the target of inactivation, we propose that MS2 is inactivated by base-catalyzed transesterification of the genome. Furthermore, given that a single genome lesion causes inactivation, the inactivation rate can be directly related to the RNA transesterification rate.

To provide further support that the mechanism underlying MS2 inactivation in our experi-

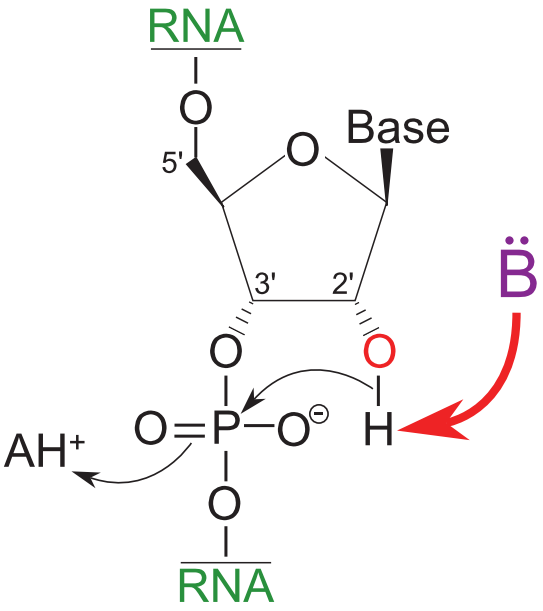


Figure 2.7: *RNA base-promoted transesterification mechanism. B denotes a base and AH⁺ an acid.*

ments was general base-catalyzed transesterification, we tested the influence of a series of bases with different pK_a values on MS2 infectivity. Specifically, by varying the base concentration at constant pH, we could determine k_j for each base according to eq. 2.2 (Figure 2.8). Log_{10} k_j was shown to be linearly correlated to the base pK_a (Figure 2.9) for all tested bases, which is consistent with an RNA transesterification mechanism.[130,131] This linear relationship can be described by the Brønsted catalysis law:[133]

$$log_{10} k_j = \beta pK_a + D \tag{2.5}$$

where the parameter β is the Brønsted coefficient, and D is a constant specific to the RNA structure and length and temperature conditions. For MS2 inactivation, we observed β = 0.41, which is consistent with RNA transesterification being a general base-catalyzed process.[134] Though this value of β was determined at 35 °C, it is not expected to change over the temperature range considered herein (see Chapter 4).

Prediction of MS2 inactivation in controlled solutions. As discussed above, each base present in solution may contribute to RNA transesterification and thereby to MS2 inactivation, according to its pK_a and activity. Rather than summarizing the contribution of non-NH_3(aq) bases to k_{obs} as $k_{background}$, we can therefore modify eq. 2.2 to include the contribution of each base in solution:

$$k_{pred} = \sum_j \{j\} k_j \tag{2.6}$$

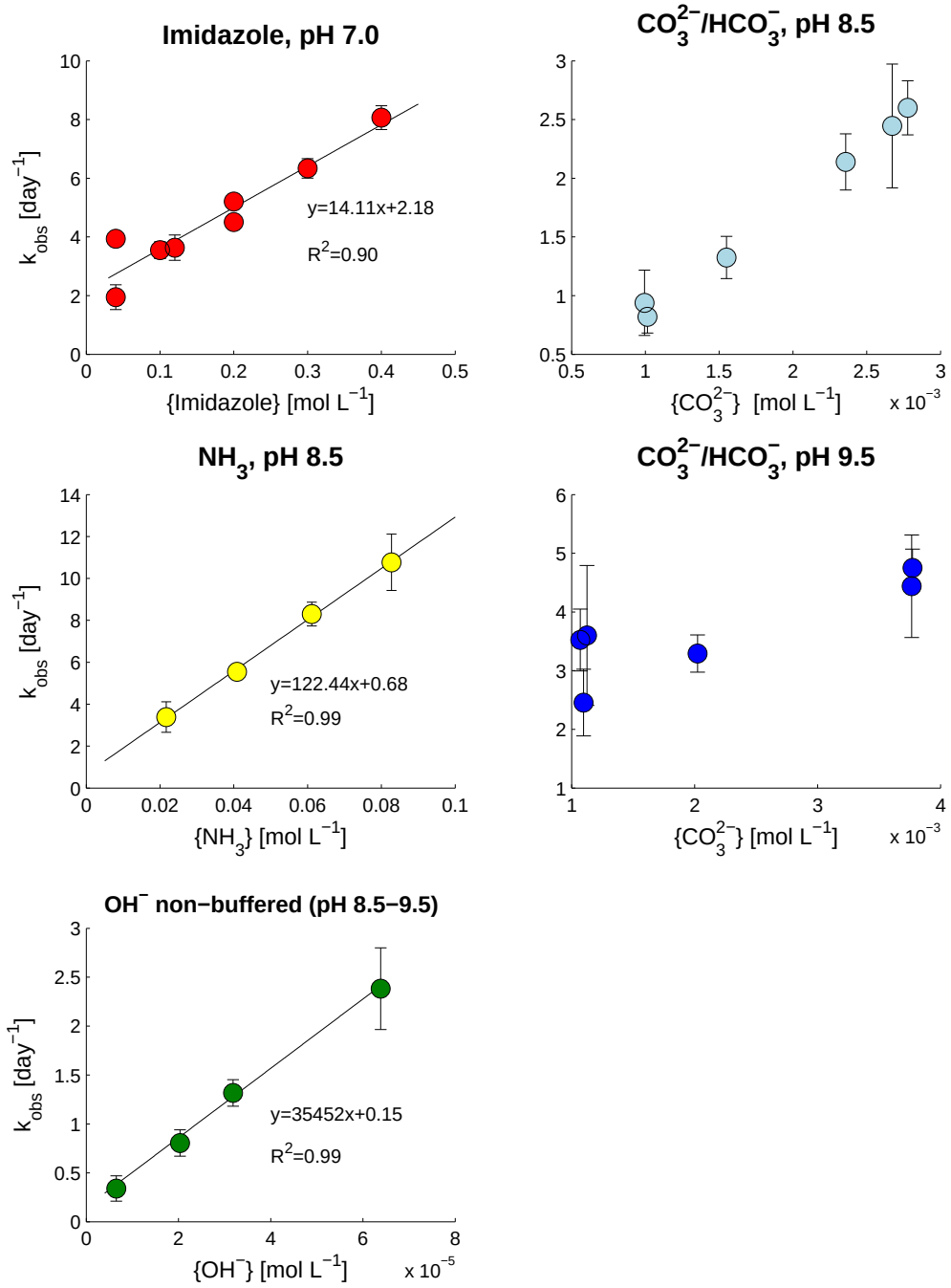


Figure 2.8: *MS2 inactivation first-order rate constant as a function of the different base activities in aqueous solutions of the pure base. For the carbonate solutions, k_{obs} were plotted against the CO_3^{2-} activity, even though actual solution contains both CO_3^{2-} and HCO_3^-. For OH^-, its activity was varied by changing the pH in a non-buffered solution with 50 mmol L^{-1} NaCl. Vertical error bars depict 95% confidence interval associated with k_{obs}.*

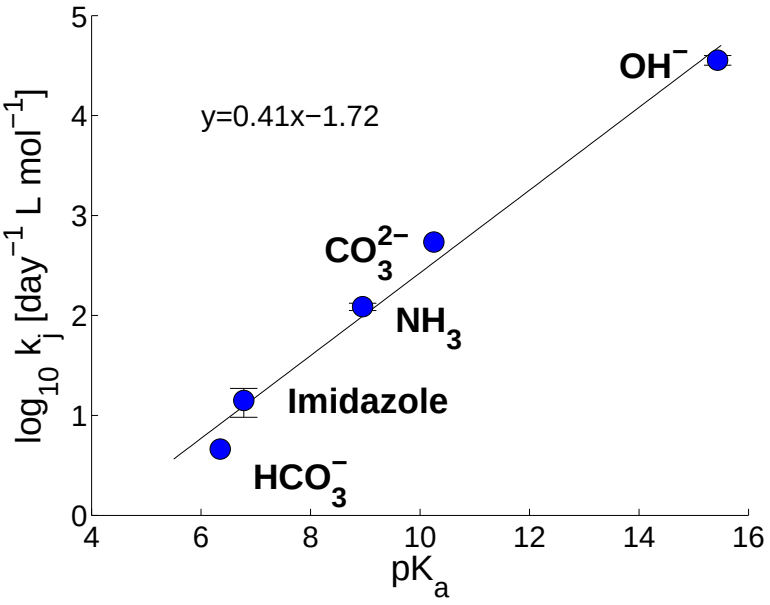


Figure 2.9: *Brønsted plot of five bases vs their second-order inactivation rate constant for MS2 at 35 °C. k_j was determined according to eq. 2.2 in aqueous solutions of the pure base (see Figure 2.9). pK_a values were corrected for temperature (see Appendix C, "Determination of pK_a (as a function of temperature (T))"). Vertical error bars depict 95% confidence intervals of k_j.*

where k_j can be determined for any base according to the Brønsted equation developed in Figure 2.9. Using eq. 2.6 we compared the predicted k_{obs} (k_{pred}) with the observed one for MS2 at 35 °C in PCa and AmCa (data shown in Figure 2.2a). k_j values derived from the Brønsted equation fitted to the experimental data (i.e., β = 0.41 and D = -1.72 ; Figure 2.9) were used for these predictions. As can be seen in Figure 2.10, eq. 2.6 accurately predicted the k_{obs} for most experimental conditions tested. A detailed look at the contribution of the different bases (Figure 2.11) revealed that NH_3(aq) was the main species contributing to inactivation in AmCa buffer for pH ≤ 9.0. Furthermore, inactivation by OH^- could explain the substantial increase in k_{obs} observed at pH 9.5 (Figure 2.2a). Carbonate species (if present) contributed to inactivation at pH ≥ 9.0, in particular in solutions with low {NH_3(aq)} (Figure 2.11). And finally, phosphate species contributed to inactivation in PCa at pH ≤ 8.0 (Figure 2.12).

Implication of the study for virus inactivation in HEAM. The behavior of viruses in typical conditions of HEAM during storage or mesophilic digestion, that is, from neutral to mildly alkaline conditions and temperature lower than 40 °C, was established to depend on the integrity of the viral genome. Specifically, loss of infectivity of ssRNA viruses could be linked to genome cleavage during base-catalyzed transesterification. This mechanism explains why DNA and dsRNA viruses have previously been found to be more resistant in HEAM than ssRNA viruses: only the ribose in single-stranded RNA configuration is susceptible to substantial transesterification.[135,136]

Inactivation of MS2 could be predicted by taking into account all bases in solution. Notably,

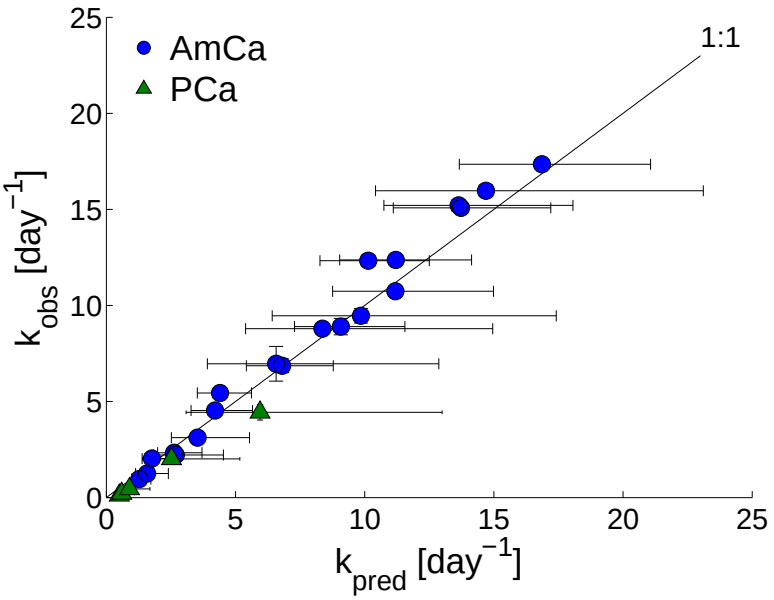


Figure 2.10: *Comparison of measured and predicted inactivation rate constants for MS2 at 35 °C. k_{pred} were determined from eq. 2.6 and with the k_j deduced from the Brønsted plot shown in Figure 2.9. Vertical error bars depict 95% confidence interval associated with k_{obs}. Horizontal error bars depict 95% confidence intervals of k_{pred}, which were calculated using the 95% CI of k_j (shown in Figure 2.9). The line represents the 1:1 ratio of k_{obs} to k_{pred}.*

this included not only NH_3(aq), but also other species commonly encountered in HEAM (e.g., carbonate, phosphate, hydroxide), though NH_3(aq) was confirmed as the dominant inactivating species at many of the conditions tested. The different bases exhibited an additive effect on inactivation. Thus, when considering measures to enhance inactivation in HEAM, for example, by adding urea,[100,110] not only the increase in pH and NH_3(aq), but also the change in the speciation of carbonate species has to be considered.

The findings of this work may be generalized to other ssRNA viruses by taking into account additional virus-specific parameters. In particular, the length of the genome is important, because inactivation by transesterification is likely a function of genome length and structure. A longer genome leads to a higher probability to form an in-line structure susceptible to cleavage by transesterification,[125] and hence should lead to faster inactivation. Correspondingly, a tendency toward faster inactivation for viruses with longer genomes was observed by Emmoth et al. in NH_3(aq) disinfection of hatchery waste.[101]

The observations of this study were made in controlled and relatively simple solutions which mimic the main features of real matrices, for example, urine, urine-feces mixtures or digested sludge. These real matrices, however, are more complex solutions which additionally contain organic compounds and metal cations, the latter being known to assist in RNA transesterification.[131,137] Therefore, the predictions of virus inactivation formulated herein will need to be challenged and confirmed in real HEAM matrices. Our data nevertheless conclusively

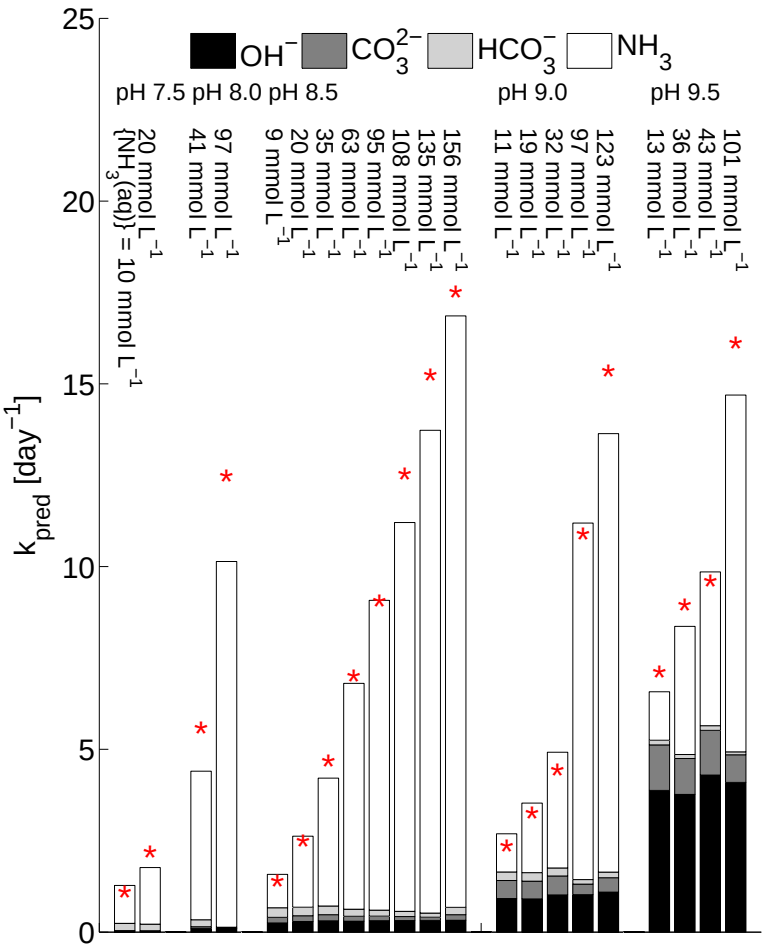


Figure 2.11: *Contribution of the different nucleophiles (bases) to the inactivation rate predicted for MS2 at 35 °C in AmCa buffer. The total added carbonate concentration was 50 mmol L^{-1}. The pH and {NH_3(aq)} are indicated in the graph. The contribution of each base is calculated from eq. 2.6. k_{obs} is depicted by *. Each bar corresponds to a data point shown in Figure 2.2a.*

demonstrate the detrimental effects of pH and NH_3(aq) on ssRNA genomes. From a practical point of view, we therefore advise to store HEAM at the highest pH, temperature and nutrient content possible in order to optimize the inactivation potential for viruses.

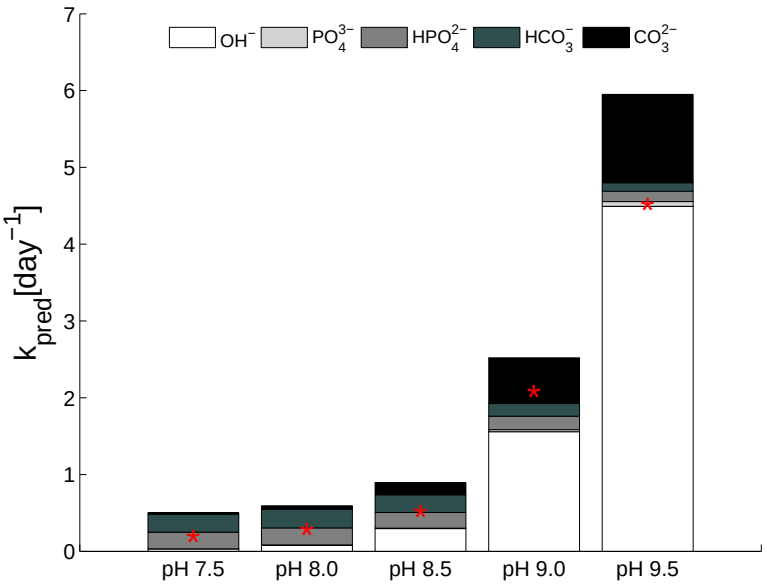
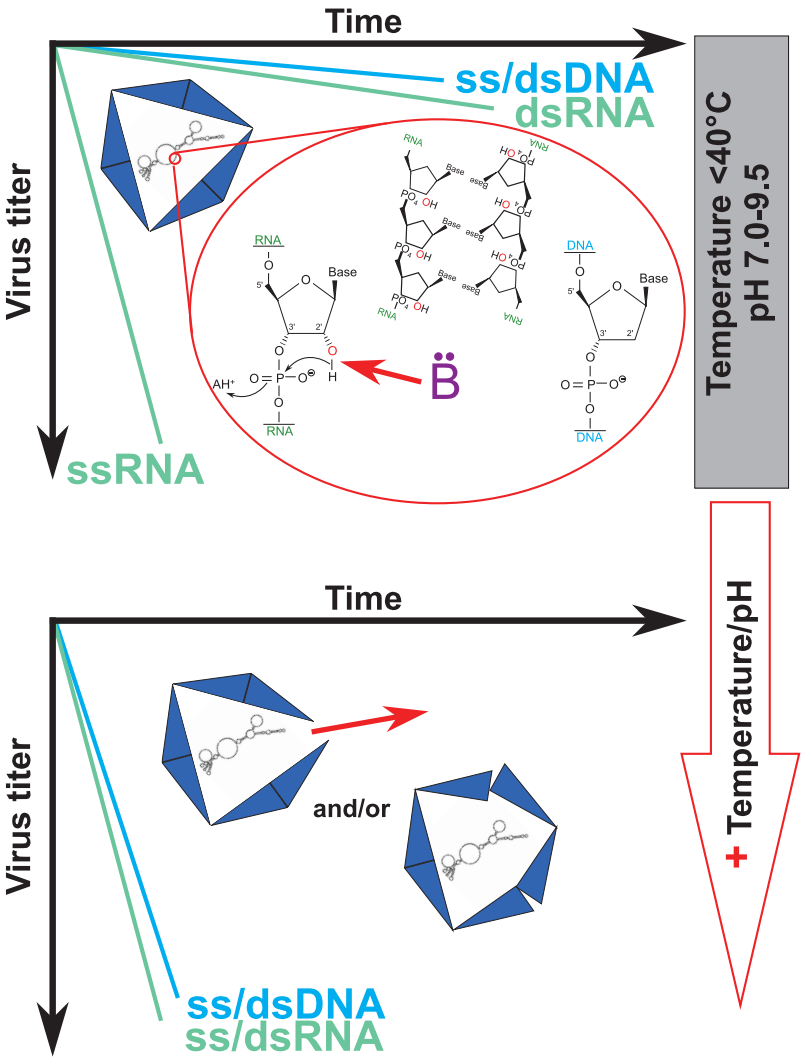


Figure 2.12: *Contribution of the different nucleophiles (bases) to the inactivation rate predicted for MS2 at 35°C in PCa buffer. The contribution is calculated from eq. 2.6. k_{obs} is depicted by *. Each bar corresponds to a data point shown in Figure 2.2a.*

3 Influence of virus genome type on viral inactivation



3.1 Introduction

Human excreta and animal manure (HEAM) may pose a threat to people because it is a major reservoir for many fecal-orally transmissible pathogenic organisms.[38] In addition, untreated waste may release undesirable compounds into the environment (e.g., macro- and micro-pollutants, heavy metals). While HEAM thus represents a source of pollution, it simultaneously is a valuable product containing nutrients e.g. nitrogen, phosphorus, potassium that are essential for plant growth[138,139] and water for irrigation.[140] 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 fecal-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.[37] Waste treatment and stabilization processes may enhance the removal or inactivation of pathogens by creating harsh environmental conditions that promote pathogen inactivation. Effective treatment of the waste could generate safe soil conditioner or fertilizer products for land application.[79] A wide array of processes is available for hygienization,[78,79] ranging from storage at ambient temperature to chemical treatment with sanitizing substances,[55,57] to aerobic or anaerobic digestion, composting, 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.[38,63] Yet, the individual and synergistic contributions of these parameters to pathogen inactivation remain to be systematically characterized.

Among possible sanitizing compounds, total ammonia (NH_4^+/NH_3) is of particular interest because it may be naturally present at substantial levels in stored urine, stored fecal sludge and anaerobically digested sludge (Table 3.1). Total ammonia is produced by urea and protein hydrolysis during storage and digestion of waste.[99,141] In its neutral, dissolved form ammonia (NH_3(aq)) is a major nitrogen source for many bacteria, eukaryotic microbes, fungi and plants. However, it may become harmful at elevated concentrations.[142] Correspondingly, NH_3(aq) was found to have biocidal activity against most pathogenic microorganisms.[94–96,101] Furthermore, NH_3(aq) was shown to be the main substances responsible for virus die-off in sludge.[90] Thus, NH_3(aq) can be seen as an in-situ sanitizer naturally present in HEAM.

The mechanisms involved in total ammonia toxicity toward eukaryotic and prokaryotic cells are not fully elucidated. Several hypotheses have been put forward to explain the biocidal effect, namely intracellular pH change, disturbance of the electrochemical gradient across the cell membrane and inhibition of enzymatic reactions.[143–146] Unlike other pathogenic microorganisms, viruses do not have a cell metabolism of their own, but rather use the host machinery to reproduce.[86] Therefore, NH_3(aq) cannot act via disrupting their metabolism, and hence the biocidal processes relevant to cells do not apply to viruses. Instead, the virucidal activity of NH_3(aq) 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_3(aq),[55,56,60,90,97,100,101,108,111,112,147–152] the mechanism of virucidal action of NH_3(aq) has received little scrutiny to date.

Unlike other living organisms, viruses carry their genetic information under different forms, specifically as single- (ss) or double-stranded (ds) RNA or DNA.[86] Several studies have observed differences in inactivation behavior among viruses with different genome types.[60,90,97,112,149–152] This suggests that the genome is an important target of NH_3(aq), and that the different genome types differ in their NH_3(aq) susceptibility. This suggestion is supported by studies using single-stranded RNA viruses, which found that genome cleavage by NH_3(aq) could explain loss of infectivity (see Chapter 2). It remains to be elucidated, however, how genome type influences inactivation kinetics and mechanisms.

Here, we exposed a suite of viruses to NH_3(aq) in controlled laboratory solutions, in order to characterize how the genome type influences the kinetics and mechanisms of NH_3(aq)-mediated inactivation. A total of seven viruses representing all genome types were studied: bacteriophages MS2 and GA and human echovirus (ssRNA); mammalian reovirus (dsRNA); bacteriophage ΦX174 (ssDNA); and bacteriophage T4 and human adenovirus (dsDNA). The viruses were exposed to synthetic buffered solution with pH of 8.0 and 9.0, temperatures of 20 and 35°C and NH_3(aq) activities ({NH_3(aq)}) of 0, 20 and 40 mmol L^{-1}. The ultimate goal of this study was to advance our understanding of the mechanisms involved in virus inactivation over a range of environmental conditions relevant to human waste storage or processing (Table 3.1).

Table 3.1: *Typical temperature, pH and NH_3(aq) conditions find in HEAM during storage and mesophilic anaerobic digestion.*

Treatment	Type of waste	Range				Ref.
		NH_4^+/NH_3 [mmol L^{-1}}]	T [°C]	pH	{NH_3} [mmol L^{-1}]	
Storage	Urine	5-449	4-35	8.2-9.8	2-246	60,153,154
	Feces (raw)	80-247	20-34	6.8-8.3	0.2-32	56,100
	Feces (+additives)	60-862	20-34	7.5-12.8	1-340	
	Feces+urine	104-1098	10-28	8.8-9.2	9-439	59
Anaerobic digestion	Animal manure	35-450	25-38	7.0-8.1	0-37	155–158
	Sewage sludge	42-45	37	7.5-8.0	1-5	150
	Activated sludge	149-191	37	7.7-7.9	7-13	159

3.2 Materials and methods

Viruses and cells. Human adenovirus type 2 (HAdV) was kindly provided by Rosina Gironès (University of Barcelona). Echovirus type 11 (EV; ATCC VR-41) and mammalian reovirus type

1 (ReoV; ATCC VR-230) were purchased from LGC Standards (Molsheim, France). HAdV, EV and ReoV were propagated on A549 human lung carcinoma epithelial cells, Buffalo Green Monkey Kidney cells (BGMK) and L929 mouse fibroblast respectively. A549 and L929 cells were kindly provided by the University Hospital of Lausanne 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^{-1}), streptomycin (20 µg mL^{-1}) (Invitrogen), and 2 or 10% fetal bovine serum (FBS; Invitrogen) and cells were incubated at 37°C in 5% CO_2 and 95% humidity. Viruses were propagated by spiking 10 µL of HAdV (10^{10}-10^{11} most probable number of cytopathogenic units (MPNCU) mL^{-1}) or 100 µL of EV or ReoV (10^{7}-10^{8} MPNCU mL^{-1}) into 160 cm^2 flasks (TPP Techno Plastic Products, Trasadingen, Switzerland) containing 95% confluent cells, and were and purified according to Bosshard et al.[160] From each flask, one mL of samples containing 10^{10}-10^{11} MPNCU mL^{-1} of HAdV, 10^{7}-10^{8} MPNCU mL^{-1} of EV or 10^{7}-10^{8} MPNCU mL^{-1} of ReoV were collected and stored at 4°C as virus stocks for the experiments. New stocks were produced before each set of experiments. Virus titers were determined by MPNCU from 5x100 µL of samples on 96-wells plates (Greiner bio-one, Frickenhausen, Germany) as described by Bosshard et al.[160] Briefly, the DMEM/MEM containing 10% FBS on a 95% confluent cell monolayer was replaced by 100 µL of virus solution and completed with 200 µl of DMEM/MEM containing 2% FBS. Cytopathogenic units could be seen after incubation times of 14, 4-7 and 10 days for HAdV, EV and ReoV, respectively. The detection limit for all viruses was 10^{2}-10^{3} MPNCU mL^{-1}.

Phages and bacteria. Coliphages MS2 (DSMZ 13767) and ΦX174 (DSMZ 4497) and their host Escherichia coli (DSMZ 5695 and DSMZ 13127, respectively) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). GA was kindly provided by Joan Jofre (University of Barcelona). GA was cultured in the same E. coli host as MS2. Coliphage T4 and its E.coli host (B1) were kindly provided by Petr Leiman (EPFL). Media used to grow E.coli B1 were free of any antibiotics. All phages were propagated and purified as described previously.[114] Stock solutions were stored in the fridge, and the same stocks were used for all the experiments. Infectivity was assessed using the double agar layer method.[115] The detection limit for all phages was 300 PFU mL^{-1}.

Chemicals. Sodium carbonate (Na_2CO_3; Fluka), ammonium chloride (NH_4Cl; Acros), potassium chloride (KCl; Acros), sodium chloride (NaCl,;Acros) and sodium phosphate (NaH_2PO_4; Acros) were used to make the experimental solutions described below.

Experimental solutions. Virus inactivation was assessed over a range of temperatures, pH and {NH_3(aq)}. Temperature, pH and {NH_3(aq)} were chosen within a relevant environmental range as detailed in Table 3.1. pH 8.0/20°C represents the baseline conditions from which pH and temperature effects were assessed. For all pH/T conditions, inactivation was quantified at {NH_3(aq)} of 0, 20 and 40 mmol L^{-1}. Phosphate carbonate buffer (PCa) was used as the NH_3(aq)-free control ({NH_3(aq)}=0 mmol L^{-1}), whereas ammonium carbonate buffer (AmCa) was used to generate solutions containing 20 and 40 mmol L^{-1} {NH_3(aq)}. The appropriate

composition of the buffers to attain a given NH_3(aq) activity was determined with PHREEQC (version 2.18.00) and a database using the Pitzer approach for calculating the ion activity, as described in Chapter 2. The exact buffer composition for each experiment can be found in the Appendix B, Table B.1).

Under baseline conditions (pH 8.0/20°C) the AmCa solutions with {NH_3(aq)}= 40 mmol L^{-1} exhibited a high ionic strength (IS) compared to the PCa solution. Therefore control experiments were conducted to distinguish between the effects of IS and NH_3(aq). These control experiments were conducted under baseline conditions in high IS PCa (PCaH), where the IS was increased by adding sodium chloride. IS was assessed by measurement of the electrical conductivity (EC) with a Cond315i conductivity meter and a TetraCon 325 probe (WTW, Weilheim, Germany). The EC corresponded to 10.44 mS cm^{-1} (PCa); 127 mS cm^{-1} (PCaH); 91.65 mS cm^{-1} (AmCa, {NH_3(aq)}=20 mmol L^{-1}) and 174.25 mS cm^{-1} (AmCa, {NH_3(aq)}=40 mmol L^{-1}). For phages MS2, ΦX174 and T4, additional experiments were conducted at pH 12.0/20°C in phosphate buffer (10 mmol L^{-1} NaH_2PO_4*H_2O) and at pH 8.0/50°C or 60°C in PCa, to assess the influence of extreme pH and temperature, respectively. Potassium carbonate solution (KCa) with K^+ activities similar to NH_4^+ in AmCa at pH 9.0 and 20°C were used to assess the effect of monovalent cations on EV inactivation.

Experimental setup. For MS2, GA, ΦX174, T4 and HAdV, one milliliter of a virus solution (10^7 -10^{10} PFU or MPNCU mL^{-1} in virus dilution buffer (VDB; 5 mmol L^{-1} NaH_2PO_4, 10 mmol L^{-1} NaCl, pH 7.5) was added to airtight 116 mL glass serum flasks (Infochroma) containing 114 mL of experimental solution. For the lower titer ReoV and EV, one milliliter of virus stock (10^6-10^8 MPNCU mL^{-1}) was added to airtight 16 mL glass serum flasks containing 14 mL of experimental buffer. After mixing, a one mL sample was taken from each flask with a sterile syringe, and was filtered through a 0.22 µm filter (Millipore). The filtered samples were directly diluted in medium containing 2% FBS (HAdV, ReoV and EV) or VDB (MS2, GA, ΦX174, T4), and were stored at 4°C for no more than six hours prior to enumeration. Each pH, T and {NH_3(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 NH_4^+/NH_3 concentration was determined by ion chromatography (ICS-3000A, IonPacCS16 column) with electrical conductivity detection (Dionex, Switzerland) and {NH_3(aq)} was calculated as described in Chapter 2.

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

$$ln\frac{C}{C_0} = -k_{obs}t \tag{3.1}$$

where C_0 and C [PFU or MPNCU mL^{-1}] are the virus concentration at time 0 (initial) and t, and k_{obs} is the first-order inactivation rate constant [day^{-1}]. The data of all replicates were pooled and the 95% confidence interval of k_{obs} was calculated from the standard error of the slope of

the pooled data.

The second-order rate constant for inactivation by NH_3(aq) (k_{NH_3}; [day^{-1} L mol^{-1}]) was determined by the best fit of a linear model according to the following relationship:

$$k_{obs} = k_{NH_3}\{NH_3\} + k_{background} \tag{3.2}$$

Where {NH_3(aq)} is the activity of NH_3(aq) and $k_{background}$ is the first-order inactivation rate constant [day^{-1}] in the absence of NH_3(aq).

Inactivation rate constants were compared by means of ANCOVA.

3.3 Results and Discussions

Virus inactivation kinetics. All viruses studied exhibited a loss of infectivity with time, and inactivation kinetics generally followed a first order model (Figures 3.1 & 3.2 and see Appendix B, Tables B.2 & B.3). Only for ReoV, poor adherence to first order kinetics was found, especially at pH8.0/20°C and at pH 9.0/20°C in PCa (see Appendix B, Table B.3).
Noticeable differences in inactivation were observed between the different viruses, which seemed driven by genome type (Figure 3.3). In PCa, the k_{obs} of the different viruses were all within one order of magnitude (see Appendix B, Tables B.2 and B.3). In the presence of NH_3(aq), however, differences between genome types were more apparent. For ssRNA viruses the presence of NH_3(aq) in solution lead to a substantial increase in inactivation kinetics, resulting in a 100 to 1000-fold greater k_{obs} compared to viruses with other genome types. The influence of genome type is further illustrated by k_{NH_3}, which was 10- to 1000-fold higher for ssRNA viruses than for viruses with other genome types (Figure 3.4 and see Appendix B, Table B.4). 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 a higher sensitivity of these viruses to NH_3(aq) compared to other viruses. Consequently, increasing {NH_3(aq)} affected ssRNA viruses more drastically than other viruses.

Even among a single genome type, differences in inactivation behavior were observed, though they were small compared to the differences between genome types. MS2 and GA showed significantly different k_{obs} ($p < 0.05$) for all conditions tested though they were of the same order of magnitude and showed the same tendency towards changes in the solution conditions (Figure 3.3 and see Appendix B, Tables B.2). EV exhibited a four-fold greater k_{obs} than MS2 at pH 8.0/20°C in AmCa and this difference was even more pronounced when increasing the temperature or pH (Figure 3.3). This is also illustrated by the stronger effect of raising temperature or pH on k_{NH_3} of EV compared to MS2 and GA (Figure 3.4).

Effect of solution conditions on inactivation.

pH. In the absence of NH_3(aq), no relevant increase in the k_{obs} of DNA viruses could be

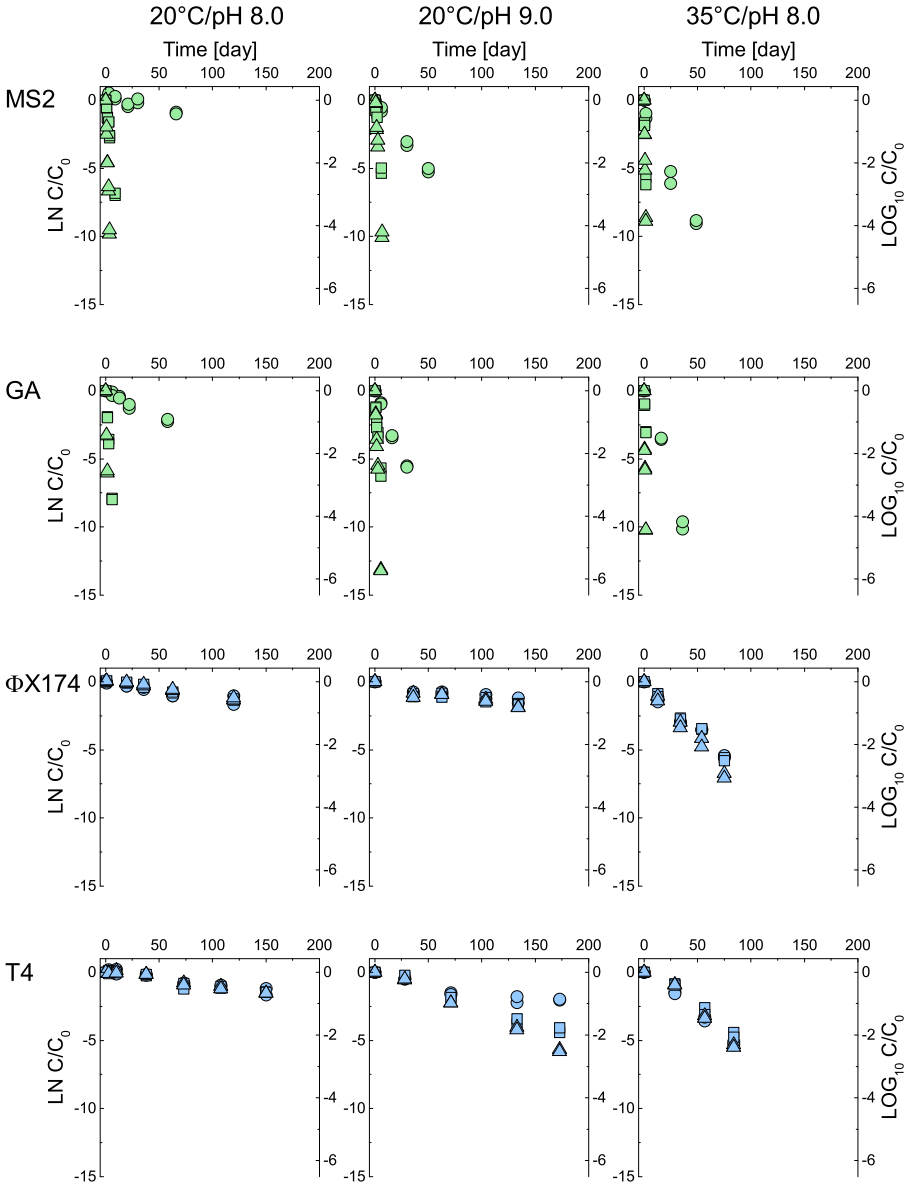


Figure 3.1: *Kinetics of phages inactivation. Circles, squares and triangles represent PCa and AmCa with an intended {NH_3(aq)} of 0, 20 and 40 mmol L^{-1} respectively. The measured {NH_3(aq)} can be found in Appendix B, Table B.1.*

observed when raising the pH from 8.0 to 9.0. Among the RNA viruses studied, MS2 and GA exhibited a significant (approximately five-fold) increase in k_{obs} (see Appendix B, Table B.2). For EV and ReoV, the scatter in the data did not allow to conclusively establish if a pH shift from 8.0 to 9.0 affected k_{obs} (see Appendix B, Table B.3). Further increase of pH to 12.0 showed that pH had a large effect on k_{obs} beyond pH 9.0 independent of the genome type, with the exceptions of ΦX174 which exhibited a high resistance to alkaline conditions (Figure

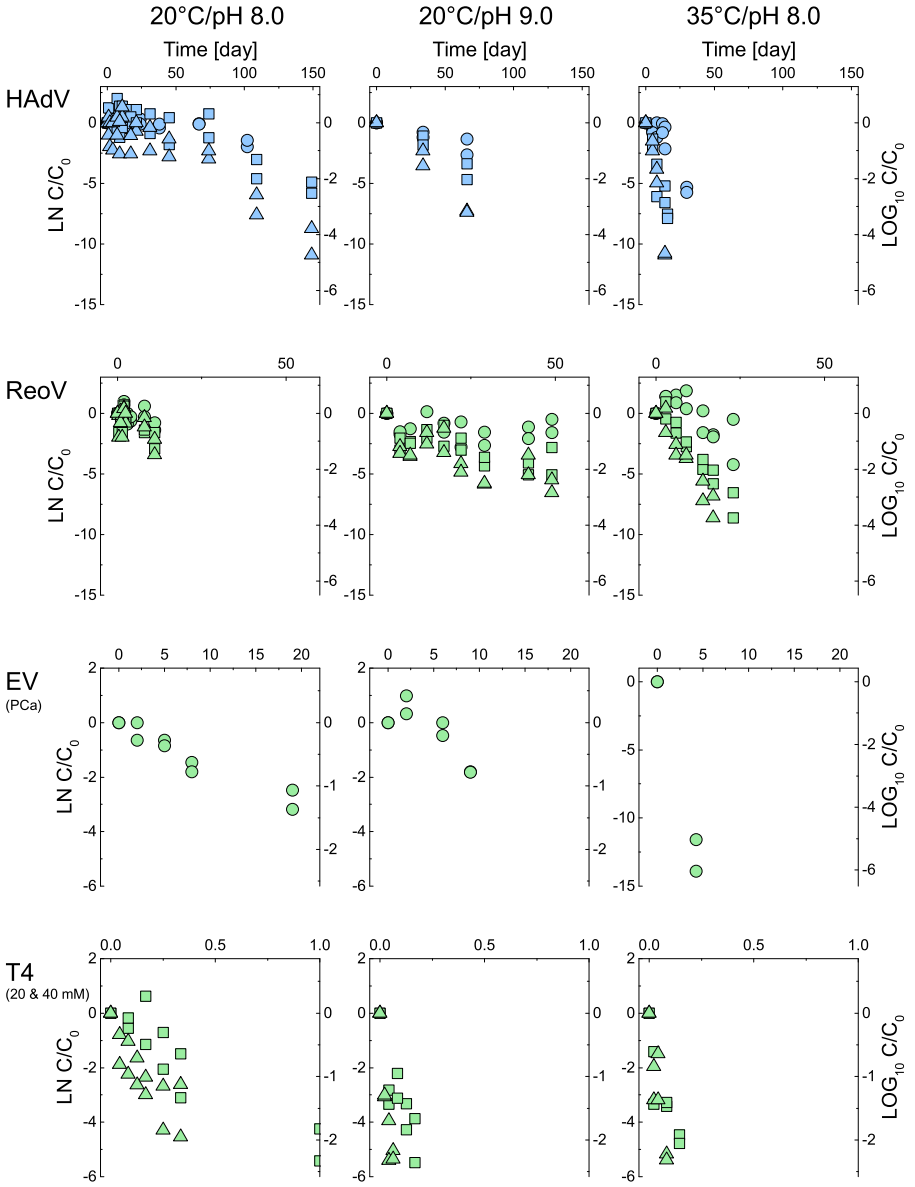
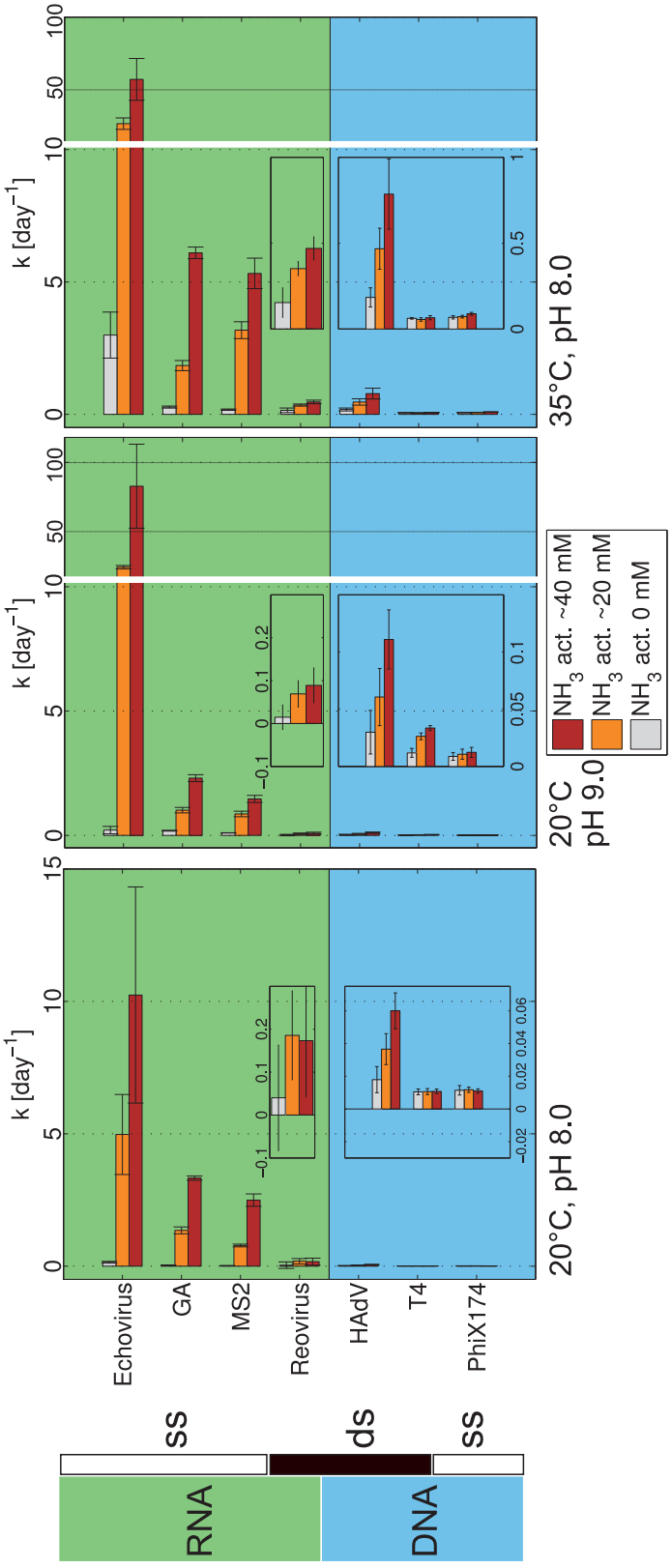


Figure 3.2: *Kinetics of mammalian viruses inactivation. Circles, squares and triangles represent PCa and AmCa with an intended {NH_3(aq)} of 0, 20 and 40 mmol L^{-1} respectively. The measured {NH_3(aq)} can be found in Appendix B, Table B.1.*

3.5). Additionally, pH did affect the susceptibility of some viruses to NH_3(aq). Most notably, the dsDNA viruses HAdV and T4 exhibited a higher k_{NH_3} at pH 9 compared to pH 8 (Figure 3.4). Similarly, the k_{NH_3} of EV increased when raising the pH from 8 to 9. For the other RNA viruses tested, however, pH did not affect k_{NH_3}.

Temperature. Unlike pH, higher temperatures led to greater k_{obs} and k_{NH_3} for all viruses tested


k [day^{-1}]
35°C, pH 8.0
20°C
pH 9.0
20°C, pH 8.0
NH$_3$ act. ~40 mM
NH$_3$ act. ~20 mM
NH$_3$ act. 0 mM
Echovirus
GA
MS2
Reovirus
HAdV
T4
PhiX174
RNA
DNA
ss
ds
ss

(see Appendix B, Tables B.2 and B.3). DNA viruses showed higher increase of k_{obs} (6-8-fold) than RNA viruses (2-4-fold), even though absolute value of k_{obs} remained low for DNA at 35°C, between 0.1 and 1 day^{-1}. The three viruses (MS2, ΦX174 and T4) tested at temperatures up to 60°C in PCa at pH 8.0 exhibited an exponential increase in k_{obs} with temperature. Interestingly, MS2 was more sensitive to inactivation by temperature compared to the two DNA phages ΦX174 and T4 up to 50°C. At 60°C, however, the gap between the k_{obs} of the three viruses drastically narrowed (Figure 3.5).

Ionic strength. Increasing ionic strength in a control solution (0 mmol L^{-1} {NH_3(aq)}) at pH 8.0 and 20°C showed to enhance inactivation of ssRNA virus, e.g. MS2, GA and EV (see Appendix B, Tables B.2 and B.3). However the effect of ionic strength on k_{obs} of ssRNA viruses remained negligible compared to the effect of {NH_3(aq)}.

Monovalent cations. Finally, for EV the effect of monovalent cations was also tested, as cations such as K^+ or NH_4^+ were implicated in promoting genome degradation via induction of nuclease activity of the virus capsid.[122–124] At pH 9.0 and 20°C, no significant increase in k_{obs} could be observed with increasing K^+ activity, whereas a comparable increase in NH_4^+ activity did enhance k_{obs} (see Appendix B, Figure B.1). We therefore attributed the observed inactivation of EV in the presence of NH_4^+ to the action of its conjugated base NH_3(aq), the activity of which is directly proportional to the activity of NH_4^+.

Causes underlying the differences in inactivation kinetics between DNA and RNA viruses. As is evident from Figure 3.3, DNA viruses are generally more stable than RNA viruses toward NH_3(aq), and among RNA viruses, the dsRNA ReoV was significantly more stable than the ssRNA viruses studied. These findings are consistent with other studies that reported DNA viruses to be more resistant to inactivation than RNA viruses in anaerobic digested sludge,[112,150] in septage sludge[151] and in stored urine.[152] This suggests that resistance to NH_3(aq) is associated with the stability of the different genome types in the presence of NH_3(aq). To account for this suggestion, the mechanisms involved in genome degradation by NH_3(aq) must be considered.

For ssRNA viruses it has previously been shown that, under the main range of temperatures (20-35°C), pH (8-9) and {NH_3(aq)} conditions tested in this study, infectivity loss can mainly be related to genome degradation (see Chapter 2). More specifically, for ssRNA phage MS2, genome degradation could be attributed to RNA cleavage via a general base-catalyzed transesterification (see Chapter 2), where the bases involved included NH_3(aq), OH^- and any other base in solution. In this process, the presence of the 2'-hydroxyl group of ribose renders

Figure 3.3 *(preceding page)*: *Comparison of k_{obs} values for all viruses tested at pH 8.0/20°C, pH 9.0/20°C and pH 8.0/35°C. The grey, orange and brown bars correspond to intended {NH_3(aq)} of approximately 0, 20 and 40 mmol L^{-1} respectively. The measured {NH_3(aq)} can be found in Appendix B, Table B.1. The error bars depict the 95% confidence intervals associated with k_{obs}.*

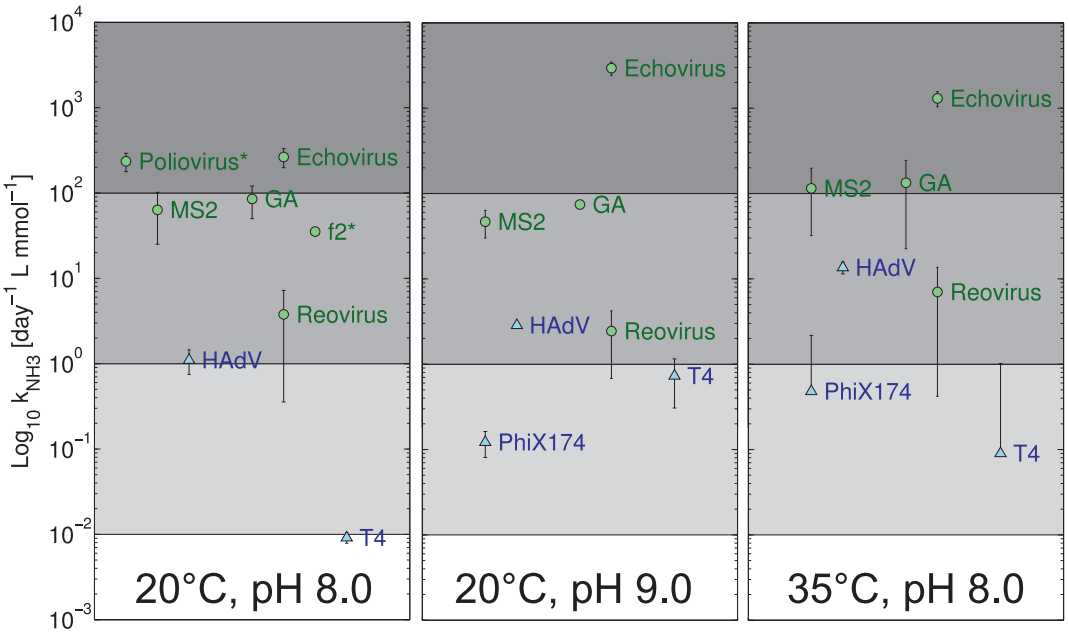


Figure 3.4: *Dependence of k_{NH_3} on temperature and pH. At pH 8.0 and 20°C, additional data (*) for f2 and poliovirus were obtained and analyzed from Burge et al.*[93] *The error bars depict the 95% confidence interval associated with k_{NH_3}. Error bars that include zero are omitted from the graph, Also omitted is the data point associated with the inactivation of ΦX174 , which was not significantly different from zero at pH 8.0 and 20°C.*

the 3',5'-phosphodiester linkages of RNA molecules susceptible to base-catalyzed transesterification (Figure 3.6).[135] This mechanism explains why ssRNA viruses are sensitive to both changes in pH and NH_3(aq), since both OH^- and NH_3(aq) can act as the base catalyst in ssRNA transesterification. In contrast, the absence of the 2'-hydroxyl group in deoxyribose protects DNA from base-catalyzed transesterification (Figure 3.6).[135] DNA viruses are therefore not sensitive to changes in pH. Interestingly, however, an increase in pH did lead to an enhanced NH_3(aq) sensitivity of DNA viruses. There thus appears to be a synergistic effect between hydroxide and NH_3(aq) that promotes DNA virus inactivation. The mechanism of this effect, however, is not understood.

While significantly slower than ssRNA cleavage, DNA cleavage can still naturally occur through the cleavage of the phosphodiester backbone, in particular at abasic sites (i.e., DNA sites lacking a purine or pyrimidine base).[161] This process is also favored under alkaline conditions; however the rate-limiting step remains the depurination/depyrimidation of the base to form the abasic site (Figure 3.7 and see Appendix B, Table B.5). Depurination was shown to be faster than depyrimidation and therefore is the critical rate in abasic site formation.[162,163] Except in the case of site-specific, self-catalyzed depurination,[164] the occurrence of which would need

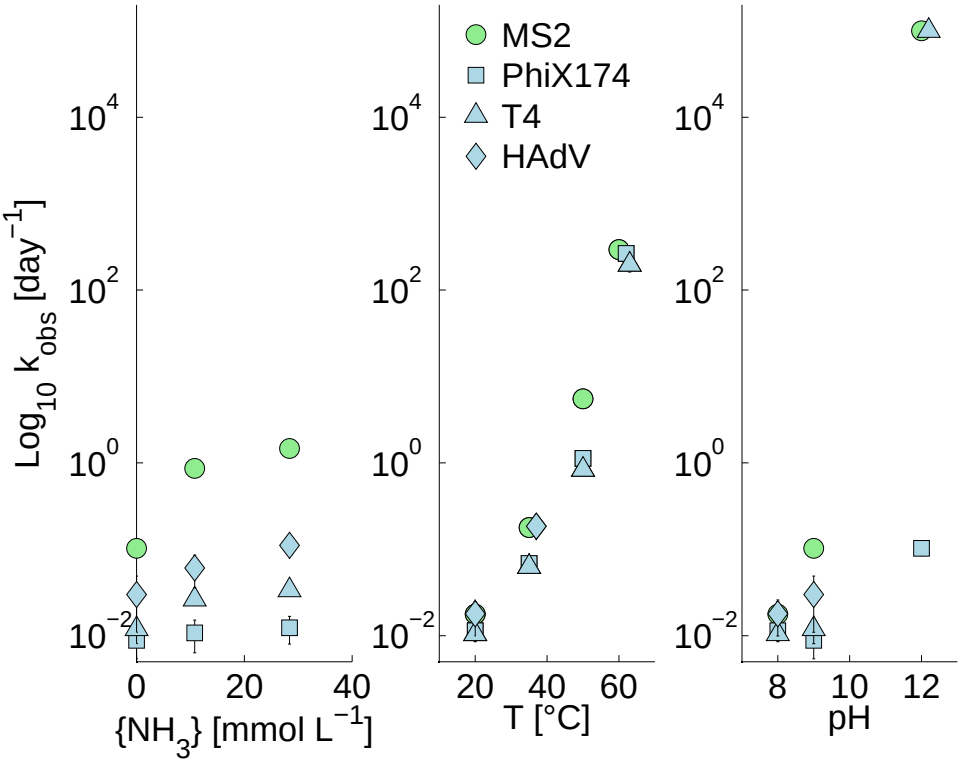


Figure 3.5: *Effect of {NH_3(aq)}, temperature and pH on k_{obs} of MS2, T4, ΦX174 and HAdV. The effect of {NH_3(aq)} was determined in PCa and AmCa buffer at pH 9.0/20°C. The effect of temperature was determined in PCa buffer at pH 8.0. The effect of pH was assessed at 20 °C in PCa buffer for pH 8.0 and 9.0 and in phosphate buffer for pH 12.0. The error bars depict the 95% confidence intervals associated with k_{obs}.*

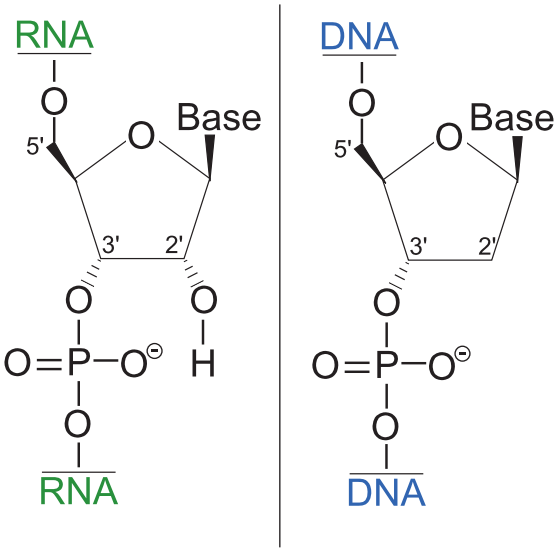


Figure 3.6: *RNA and DNA structure.*

to be verified for the DNA viruses studied herein, spontaneous depurination proceeds at an approximately 100-fold slower rate at pH 7.0 and 37°C compared to RNA cleavage (Figure 3.7 and see Appendix B, Table B.5). This slower rate of genome degradation explains why DNA viruses are more resistant to inactivation by NH_3(aq) than RNA viruses under the conditions of this study (Figure 3.3).

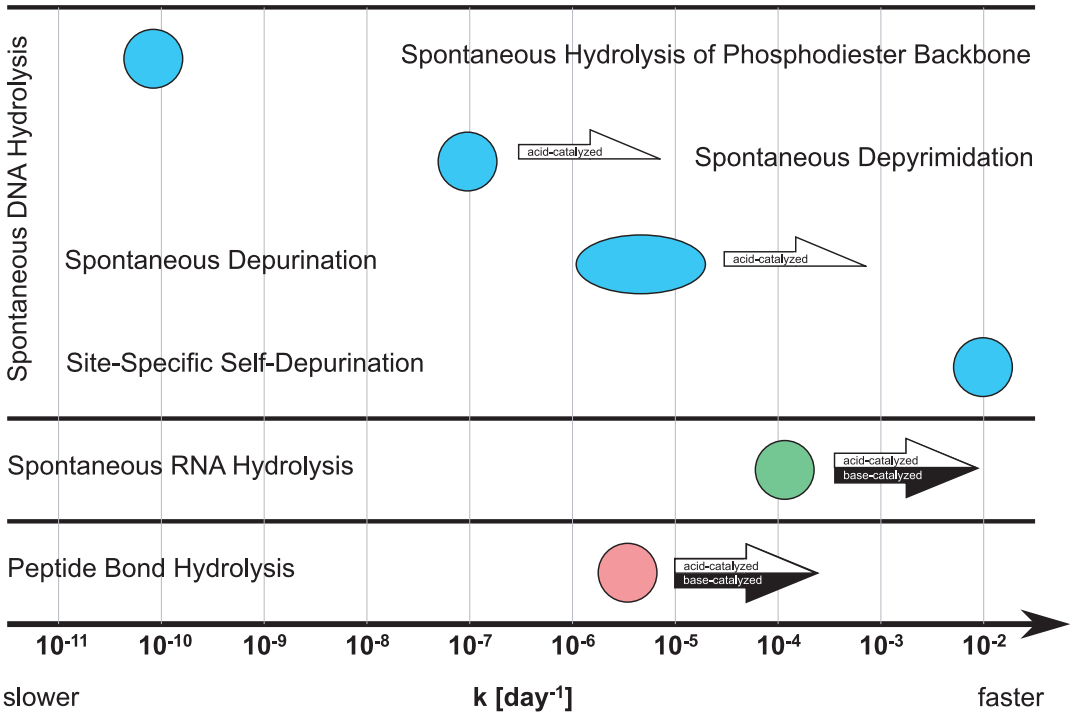


Figure 3.7: *Comparison of DNA, RNA and protein cleavage rate constants. Data refer to the hydrolysis reaction (cleavage due to reaction with water) at pH 7.0, 37°C, and are obtained from literature (references and exact numbers, see Appendix B, Table B.5). Except for spontaneous hydrolysis of phosphodiester backbone which was measured at pH 7.0, 25°C. The arrows indicate the direction of the cleavage rate constants in the case of acid and/or basic conditions.*

Besides genome degradation, virus inactivation can involve degradation of the viral proteins. As was established in Chapter 2, protein degradation does not contribute significantly to inactivation of ssRNA viruses by NH_3(aq). This can be explained by the rapid rate of ssRNA cleavage, which is much faster than peptide bond cleavage (Figure 3.7). In the case of DNA viruses, however, the situation may differ. At conditions similar to those investigated herein (pH 7.0 and 37°C) the slower DNA cleavage occurs at a rate similar to that of peptide bond cleavage (Figure 3.7 and see Appendix B, Table B.5). Thus, the inactivation of DNA viruses likely involves both genome and protein damage respectively in neutral pH conditions. A shift to more alkaline conditions increases RNA and peptide bond cleavage, which are both base-catalyzed processes,[119,126,131,165] but not the rate limiting step of DNA cleavage, depurination, which is an acid-catalyzed process.[166] Even though it is a base-catalyzed process, peptide

bond cleavage was shown to remain slower than RNA cleavage when increasing pH and temperature. However when increasing the pH, the rate of peptide cleavage will increase while DNA cleavage will decrease. Therefore, DNA virus inactivation should shift from genome- and protein-mediated inactivation to mostly protein-mediated inactivation in more alkaline conditions. This could explain why we observed some heterogeneity among the inactivation kinetics of different DNA viruses (Figure 3.3, Figure 3.4): HAdV, T4 and ΦX174 possess a few dozen different structural proteins which likely offer different extents of protection from protein cleavage (such as the proteins forming the capsid of the extraordinarily pH-resistant ΦX174; Figure 3.5), and hence lead to differences in inactivation kinetics.

Causes underlying differences in inactivation among RNA viruses. Relevant differences in inactivation kinetics were not only observed between DNA and RNA viruses, but also among RNA viruses. A likely cause for this observation is the complex structure of ssRNA. Any ssRNA forms higher-ordered structures through base pairing and tertiary interactions.[167] Among these structures, single-stranded regions are more likely to adopt in-line conformations. In-line conformations were shown to be more susceptible to cleavage by base-catalyzed transesterification, because they sterically allow for attacks of the phosphorus by the 2'-hydroxyl group of the adjacent nucleotide (Figure 3.6).[125] In comparison, highly structured and folded regions are locked into positions and therefore less likely cleaved. Thus, heterogeneity among ssRNA virus towards inactivation in the conditions of this study may be explained by the difference in sequence and length of the genome which further determined its structure. This accounts for the fact that similar viruses with the same genome size (e.g. MS2 and GA with a 3570 bases genome) but dissimilar genome sequence exhibited different inactivation inactivation rate constants. In the case of EV, the length of the genome (7440 bases genome) may be responsible for more rapid inactivation observed under all conditions tested, 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) are consistent with reports by others. At pH 8.0/20°C, Burge et al.[93] observed similar differences between phage f2 and poliovirus (Figure 3.4). Furthermore, the susceptibility of ssRNA viruses towards NH_3(aq) during disinfection of hatchery waste was shown to be positively correlated with viral genome length (Figure 3.8).[101] The longer genome of EV however fails to explain the very fast inactivation compared to MS2 and GA at pH 9.0/20°C or pH 8.0/35°C (Figure 3.3 and Figure 3.4). As discussed in Chapter 2, monovalent cations such as NH_4^+ or K^+ have been postulated to enhance endonuclease activity exerted by the viral capsid, causing additional genome degradation. However, this process could neither be confirmed for MS2 (see Chapter 2) nor for EV, as K^+ failed to promote inactivation (Figure B.1). The reason for the great sensitivity of EV toward NH_3(aq) remains therefore 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 NH_3(aq) inactivation than its single-stranded homologs. Similar trends were reported by others including Ward et al.,[90] who also observed that ReoV was more resistant than other ssRNA

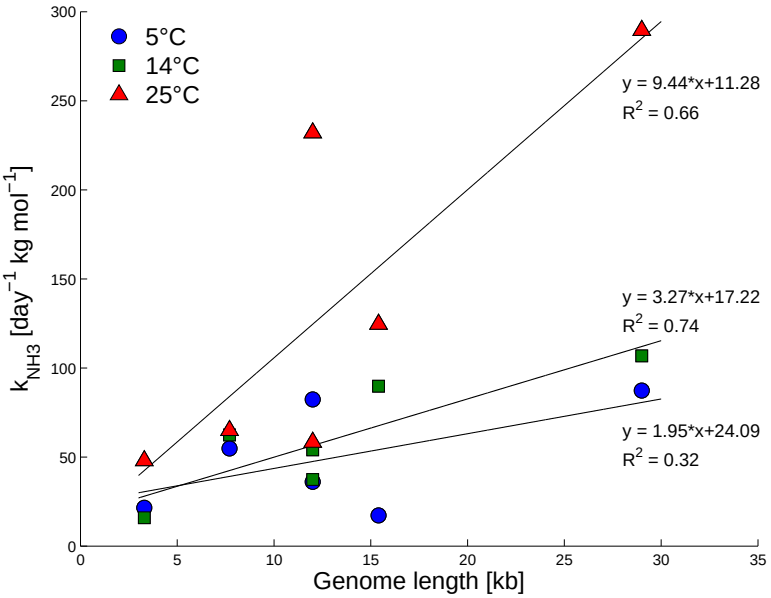


Figure 3.8: *k_{NH_3} for ssRNA viruses as a function of the genome length at different temperatures. Values for k_{NH_3} were calculated based on the k_{obs} reported by Emmoth et al.[101] The following genome length were assumed: MS2: 3.6 kilo-base (kb); feline calicivirus: 7.7 kb; avian influenza 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 a given temperature conditions and k_{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 virus. Note that the concentration of NH_3 was reported im mol per kg of treated hatchery waste.*

viruses, i.e. Polio-, Coxsackie- and Echovirus, in anaerobically digested sludge. Furthermore, Hoglund et al.[97] reported no relevant enhancement in the inactivation of the dsRNA rhesus rotavirus in stored urine compared to the NH_3(aq)-free control solution. A likely explanation for the resistance of dsRNA toward NH_3(aq) lies in its configuration. Usher[136] 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 (Figure 3.6).[125] This was later experimentally observed with single-stranded RNA of which double-stranded domains were more resistant to cleavage than single-stranded regions.[168–170] Finally, Burge et al.[93] 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 RNA viruses.

Inactivation mechanisms under extreme pH and temperature conditions. One way to enhance virus inactivation in waste is to push the system toward even harsher pH and temperature conditions than those naturally encountered in waste. To further investigate the dependence of virus inactivation on genome type under such extreme conditions, experi-

ments were conducted in the absence of NH_3(aq), but at elevated pH (up to 12) or temperature (up to 60°C). Under these extreme conditions, the inactivation kinetics of ssRNA and DNA viruses no longer exhibited major differences (Figure 3.5), with the notable exceptions of ΦX174 at pH 12.0. Thus, RNA cleavage was likely no longer the rate determining process in MS2 inactivation. . As discussed above neither peptide bond nor DNA cleavage increase sufficiently with pH or temperature to account for the similar inactivation kinetics of DNA and ssRNA viruses. Instead, we hypothesize that protein denaturation is the main mechanism responsible for inactivation under extreme conditions. Changes in the viral proteins' secondary, tertiary and quaternary structure may result from disruption of hydrogen bonds, disulfide bridges, ionic interactions and van der Waals dispersion forces by heat or alkaline pH. Viral protein denaturation can ultimately lead to the loss in viral protein functions such as host attachment, genome delivery and genome protection . Aitken et al.observed a high activation energy characteristic of protein denaturation for poliovirus inactivation in biosolids under thermophilic anaerobic conditions (49-53 °C).[171] Nuanualsuwan and Cliver 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.[172] Dimmock showed that RNA infectivity was thermally reduced at the same rate as poliovirus and rhinovirus infectivity between 20-40°C but was far more stable for temperatures between 40-60 °C, whereas the opposite was observed for antigenic determinants indicative of protein integrity.[173] Furthermore, it was observed that poliovirus RNA, but not rhinovirus RNA, became sensitive to RNase after exposure to temperatures between 40-60 °C.[173] Sensitivity to RNAse could be caused by the release of the genome from the protective capsid. This process was observed upon heating poliovirus at 42-45°C[174] or exposing it to alkaline pH at 40°C.[120] Genome release was found to occur at a pH between 8.0-9.0 for a ssRNA insect virus[175] and at a temperature between 50-70°C at pH 7.0 for dsDNA phage lambda and HK97[176,177] and ssRNA poliovirus.[178] Specifically, Duda et al.[176] reported a rate constant of around 10^2 day^{-1} for the release of HK97 genome from capsid at 65°C. Alternatively, genome sensitivity to nucleases could result from enhanced access of the enzyme to the genome within the capsid. This was observed by Cotmore et al., who determined that minute mice viruses (ssDNA, Parvoviridae), retained their antigenic determinants, and hence their protein integrity, up to 70°C despite an increasing access to the genome by external nuclease.[179]

Whether protein denaturation causes inactivation through loss of host attachment, inhibition of genome delivery or genome release or a combination of these viral functions remains unclear and is very likely virus-dependent. However, it can be concluded that inactivation under extreme conditions appears to be protein-related and therefore independent of genome type. This can explain the similar inactivation rate constants we observed among different viruses under extreme conditions (Figure 3.5). While our data as well as other studies[150,180] thus showed a reduction of the heterogeneity among viruses under extreme conditions, other work has demonstrated that some heterogeneity in virus inactivation kinetics is conserved even under high pH and temperature conditions.[93,111,181–183] Very resistant viruses include phage lambda[184] and ΦX174 (our study) for high pH and parvovirus and salmonella phage

28B for high temperature.[112,185–188] The reasons however of these resistant behaviors remain unknown.

Implication of this study for virus inactivation in natural/complex matrices. This work suggests that virus inactivation kinetics heterogeneity is mainly driven by 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 driven by protein structure and stability. Thus, virus genome type is of less importance during alkaline stabilization or thermophilic digestion whereas it is a key parameter determining virus fate during HEAM storage or mesophilic digestion. Consequently, DNA and dsRNA viruses, e.g. adenovirus or rotavirus respectively, can be expected to be the most persistent in the latter treatments. The kinetic trends observed in this study were generally confirmed in studies using real matrices,[90,97,112,150–152] though some work reported contradictory results.[60,100,148] This may result from the fact that real matrices are more complex that the well-controlled solutions used in this study. Our conclusions were drawn from experiments conducted in homogeneous matrices where only few parameters, considered as the main ones, were controlled. Real matrices, like sludge or urine, however, may contain additional inactivating factors. For example, the presence of metal ions may enhance inactivation, especially in the case of ssRNA virus, by accelerating RNA base-catalyzed transesterification.[131] Detergents were also shown to reduce and increase inactivation of enterovirus and reovirus respectively.[189] Furthermore, in the case of sludge, viruses may adsorb to the solid fraction and thereby be protected from the inactivating agents present in the bulk.[190] The water content may be an important parameter as well, determining the mobility and concentration of inactivating or protective agents and water evaporation itself can lead to virus inactivation during dewatering process.[70,71] Finally, the microbial or related enzyme activity may also enhance inactivation during sludge digestion.[66–69]

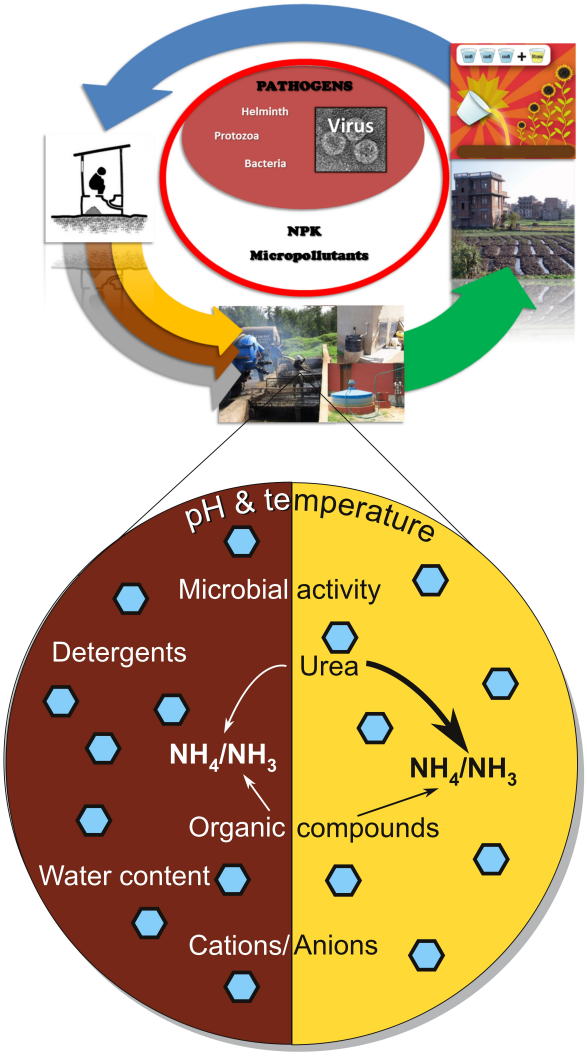
The conclusions of this study were drawn from experiments made on naked viruses. However, enveloped viruses are expected to behave differently due to the instability of the lipid bilayer in alkaline conditions. It was observed that phospholipids were cleaved at a rate of ~ $1*10^{-2}$ day^{-1} at 40°C and pH 9.0,[191] which is close to the RNA cleavage rate constant for similar conditions ($1.6*10^{-2}$ day^{-1} at pH 9.0 and 37°C).[126] Consequently, Elving et al.[192] showed that MS2 was more persistent than the enveloped dsRNA enveloped phage Φ6 and the enveloped ssRNA influenza A virus during composting of manure at 35-55°C, whereas Emmoth et al.[101] did not observe a significant influence of the envelope among ssRNA virus inactivated in manure at 4-25°C.

Concerns about fecal-oral transmission of emerging viruses such as Coronavirus (SARS)[193,194] or Filovirus (Ebola)[195] may be partially quelled from the results of this study. The fact that they are ssRNA virus which, in addition, 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 NH_3(aq) are reached.

Finally, based on the results of this study and work by others, we can issue recommenda-

tions regarding the use of indicators for the inactivation of actual human viruses in waste. Specifically, for human waste treated in mildly alkaline conditions (pH 7.0-9.5) and moderate temperature (4-35°C), we suggest the use of MS2 as conservative indicator to assess the inactivation of ssRNA viruses as Emmoth et al.[101] previously, and the very stable ΦX174 or dsDNA phages (T-phages, PRD1 or salmonella phage 28B) as indicators for persistent viruses. For higher pH and temperature conditions, however, the use of very resistant viruses such as ΦX174 or parvovirus as worst case versus more sensitive but more representative phages remains to be discussed.

4 Virus inactivation in urine and sludge under typical conditions of storage or mesophilic digestion



4.1 Introduction

Prior to reuse or discharge, human excreta and animal manure (HEAM) need to be correctly managed to avoid the introduction of pollutants (macro- and micro-pollutants) and pathogens into the environment.[38,47] If adequately treated, however, environmental pollution and public health issues resulting from the disposal of waste can be minimized.[33] Within the framework of water, sanitation and hygiene, organized excreta containment, collection and treatment is one of the pillars in breaking the transmission cycle of fecal-orally transmitted pathogens.[1] Since currently only a low percentage of the waste collected is safely managed in most cities in developing countries, on-site storage or treatment offer an opportunity to reduce the pathogen load before disposal.[52] On-site treatment should in particular focus on viruses and helminths, since they were shown to be the most persistent pathogens in excreta, in particular during storage at ambient temperature.[59,60,100]

In previous work, we characterized virus inactivation in well-controlled laboratory solutions under conditions of pH, temperature and chemical composition typically encountered during storage or mesophilic digestion (Chapter 2 and 3). As the next step, the current study aims to extend our understanding of virus inactivation to real matrices associated with on-site waste storage (sludge, stored urine). Compared to the sterile, well-controlled solutions used in our previous studies, real matrices exhibit a higher level of complexity: they contain particles, additional chemical constituents that were not previously tested (e.g., metals or organic acids), as well as live communities of microorganisms that may contribute to virus inactivation. It therefore remains to be established if the drivers of virus inactivation determined in well-controlled solutions also apply to real conditions.

In well-controlled solutions, single-stranded (ss)RNA viruses were shown to be sensitive to pH and the presence of bases in solution, whereas double-stranded (ds) RNA and both ssDNA and dsDNA viruses exhibited high survival rates under the same conditions (Chapter 3). The main factor underlying these differences was the lability of the ssRNA genome under the conditions encountered in stored urine or sludge. More specifically, inactivation resulted from base-catalyzed transesterification of the ssRNA genome, which causes the genome to cleave and renders the virus inactivated (Chapter 2). In well-controlled solution, the most important bases catalyzing this reaction are hydroxide and ammonia, though other bases such as bicarbonate also contribute. Based on this mechanistic insight, a model to estimate the inactivation rate constant under typical urine and sludge storage conditions was established for the indicator ssRNA phage MS2. Using the solution composition as the input, this model was able to accurately estimate the MS2 inactivation rate constant at 35°C and over a pH range of 7.0-9.5.

The overall goal of this work was to determine if the principles of virus inactivation established in well-controlled solution apply to real HEAM matrices. Specific objectives were i) to validate the previous approach to estimate MS2 inactivation for the more complex conditions encountered in real HEAM, i.e., over a greater temperature range 4-35°C and in real urine

and sludge matrices; and ii) to establish if the trends observed for the inactivation of viruses with different genome types in laboratory solutions correspond to those in real matrices. To reach these objectives, the pH, temperature and ion composition of different (diluted) stored urine and sludge solutions was determined, and MS2 inactivation in these solutions was monitored and compared to the predicted inactivation. Secondly, the inactivation kinetics of the base-sensitive ssRNA phage MS2 as well as the more resistant DNA phages T4, ΦX174 and the dsDNA human adenovirus (HAdV) were determined in stored urine and sludge, and were compared to results from controlled laboratory studies. Finally, experiments were conducted to determine if factors specific to stored urine and sludge, such as microbial activity or the presence of metals, influence inactivation.

4.2 Materials and methods

Virus and cells. HAdV type 2 was kindly provided by Rosina Gironès (University of Barcelona. HAdV was propagated on A549 human lung carcinoma epithelial cells, kindly provided by the University Hospital of Lausanne. A549 cells were cultivated in high-glucose, pyruvate Dulbecco's modified Eagle's medium (DMEM; Invitrogen). The media was supplemented with penicillin (20 U mL^{-1}), streptomycin (20 µg mL^{-1}) (Invitrogen), and 2 or 10% fetal bovine serum (FBS; Invitrogen) and cells were incubated at 37°C in 5% CO2 and 95% humidity. Viruses were propagated by spiking 10 µL of HAdV (10^{10}-10^{11} most probable number of cytopathogenic units (MPNCU) mL^{-1}) into 160 cm^2 flasks (TPP Techno Plastic Products, Trasadingen, Switzerland) containing 95% confluent cells, and were purified according to Bosshard et al.[160] From each flask, one mL of samples containing 10^{10}-10^{11} MPNCU mL^{-1} of HAdV was collected and stored at 4°C as virus stocks for the experiments. New stocks were produced before each set of experiments. Virus titers were determined by MPNCU from 5x100 µL of samples on 96-wells plates (Greiner bio-one, Frickenhausen, Germany) as described by Bosshard et al.[160] Briefly, the DMEM containing 10% FBS on a 95% confluent cell monolayer was replaced by 100 µL of virus solution and completed with 200 µL of DMEM containing 2% FBS. Cytopathogenic units could be discerned after an incubation time of 14 days. The detection limit for all viruses was 10^2-10^3 MPNCU mL^{-1}.

Phages and bacteria. Coliphages MS2 (DSMZ 13767) and ΦX174 (DSMZ 4497) and their host Escherichia coli (DSMZ 5695 and DSMZ 13127, respectively) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Coliphage T4 and its E.coli host (B1) were kindly provided by Petr Leiman (EPFL). Media used to grow E.coli B1 were free of any antibiotics. All phages were propagated and purified as described previously.[114] Stock solutions were stored in the fridge, and the same stocks were used for all the experiments. Infectivity was assessed using the double agar layer method.[115] The detection limit for all phages was 300 PFU mL^{-1}.

Stored urine and sludge. Undiluted stored urine was obtained from the Swiss Federal Institute of Aquatic Sciences (Eawag) in Dübendorf, Switzerland (CH), where urine is collected from

waterless urinals and men's NoMix toilets and women's NoMix toilets respectively. Three batches of male urine taken in 3 different years (2012, 2013, 2014) and one batch of female urine (2014) were obtained and used for experimentation. An additional batch of stored urine was collected from urine diverting dry toilets in Durban, South Africa (SA) in 2014. Diluted urine was obtained by mixing undiluted urine and MilliQ water at urine:water ratios of 1:1, 1:2 and 1:9. In total, the various urine batches and dilutions yielded 15 urine samples (U1-U15; Table 4.1 and see Appendix C, Table C.1). For experiments with sludge, two batches of sludge were used: the first batch (S1) consists of a synthetic fecal sludge made out of walnuts, straw flour, kaolinite, sodium phosphate, ammonium chloride and potassium nitrate according to Gallandat et al.,[196] stabilized after digestion during a few days by an inoculum of bacteria obtained from a thermophilic anaerobic digester. The second batch (S2) consisted of fecal sludge collected from septic tanks in Switzerland.

Table 4.1: *Stored urine and sludge characteristics.(Part1)*

ID	Sample description			T [°C]	pH	EC [mS cm^{-1}]	{NH_3} [mmol L^{-1}]		Virus tested
								dilution	
U1	Urine	CH	male, 2012	20	8.47		15.8	1:0	MS2
U2					8.49		9.6	1:1	MS2
U3					8.45		1.9	1:9	MS2
U4				35	8.15		19.1	1:0	MS2, HAdV
U5					8.19		24.6		MS2
U6					8.19		24.4		MS2
U7					8.22		13.6	1:1	MS2
U8					8.15		12.2		MS2
U9					8.19		8.4	1:2	MS2
U10					8.13		2.5	1:9	MS2
U11					8.13		2.7		MS2
U12			male, 2013	35	8.72	33.6	81	1:0	MS2, HAdV, ΦX174, T4
U13			male, 2014	35	8.79	33.0	106	1:0	MS2
U14			female, 2014	35	8.49	16.0	28.2	1:0	MS2
U15		SA	2014	35	8.48	33.6	71.1	1:0	MS2
								%TS	
S1	Sludge	CH	Synthetic	35	8.24		27.7	3.3	MS2
S2		CH	Septic tank	35	7.76		1.3	6.7	MS2, HAdV, ΦX174, T4

Upon arrival in the lab, stored urine and sludge was stored at 4 °C until use. Prior to characterization, all urine samples were centrifuged at 10'000xg for 10 minutes. For sludge, 5-10 ml of milliQ water were added, and the sample was shaken for 10-15 minutes and centrifuged at 4000xg for 15 minutes. Urine and sludge physical and chemical characteristics were determined as follows: pH was measured at experimental temperature (780 pH Meter with primatrode with NTC no. 6.0228.010, Metrohm, Herisau, Switzerland)(note that the urine pH of our samples were rather low compared to other studies, see Chapter 3, Table 3.1); the total ammonia (NH_4^+/NH_3) concentration was determined by ion chromatography (ICS-3000, IonPacCS16 column) with electrical conductivity detection (Dionex, Switzerland); phosphate,

sulfate and chlorine concentrations were measured by ion chromatography (ICS-3000, IonPac AS11-HC column); magnesium, calcium, potassium and sodium by inductively coupled plasma optical emission spectrometry (ICP-OES, Ciros, Spectro Analytical Instruments, Kleve, Germany); soluble chemical oxygen demand (SCOD) with cuvette tests (Hach-Lange, Berlin, Germany) and total inorganic carbon (TIC) by means of a TOC-TN Analyser (IL 550, Hach-Lange, Berlin, Germany). For NH_4^+/NH_3 and TIC measurements, samples were diluted in 0.01 M HCl and NaOH respectively to avoid loss of NH_3 and CO_2 respectively. For all other ions, samples were diluted in MilliQ water. Additionally, total solid (TS) were determined in sludge according to standard methods[197] and, given the low TS (<7%), one Liter of sludge was considered as one kg. This characterization was generally performed in quadruplicate for urine and in triplicate for sludge (see below for more details). For male urine (U1-U11, see Table 4.1 and Appendix C, Table C.1), the ion content was measured once at room temperature, and only pH and NH_4^+/NH_3 were determined under each experimental condition.

Ion activities in each matrix were determined as a function of experimental temperature, pH and solution composition using PHREEQC (version 2.18.00) and a database using the Pitzer approach for calculating ion activities. Note that the concentration expressed in mol kg^{-1} in PHREEQC were considered equivalent to mol L^{-1}. SCOD was transformed to acetate equivalents, because acetate was shown to represent 47% of the SCOD in stored urine.[198] The transformation was determined according to the following stoichiometric relation:
$C_2H_3O_2$+1.75CO_2→ 2CO_2+1.5H_2O

Experimental setup. *Φ*X174 and T4 were tested in one batch of urine and one batch of sludge, HAdV in two batches of urine and one batch of sludge, whereas MS2 in all the batches described in Table 4.1. For urine, one milliliter of a MS2, *Φ*X174, T4 and HAdV solution containing 10^7 -10^{10} PFU or MPNCU mL^{-1} in virus dilution buffer (VDB; 5 mmol L^{-1} NaH_2PO_4, 10 mmol L^{-1} NaCl, pH 7.5) was added to airtight 116 mL glass serum flasks (Infochroma) containing 114 mL of urine solution stored at the targeted temperature for at least one day. After mixing, a one mL sample was taken from each flask with a sterile syringe, was filtered through a 0.22 µm filter (Millipore), and was enumerated. For sludge, one mL of a MS2, *Φ*X174, T4 and HAdV solution (10^7 -10^{10} PFU or MPNCU mL^{-1} in VDB) was added to 150-300 mL of sludge stored at the targeted temperature for at least one day. The suspension was stirred during 5 minutes and was then distributed in 15 mL falcon tubes (Sarstedt, Nümbrecht), which were tightly closed with cap and parafilm. Two grams of (sacrificial) samples were collected, weighed and mixed with 10 mL beef extract solution (BES; 100 g L^{-1} beef extract (Merck), pH 7.2) to promote the elution of viruses from solids. Samples were then shaken for 10-15 minutes and centrifuge for 20 minutes at 4000xg at room temperature.[149] Preliminary experiments showed 95% recovery of MS2 spiked in sludge with this procedure. One mL of the supernatant was then taken with a sterile syringe and filtered through a 0.22 µm filter (Millipore). The filtered samples were directly diluted in medium containing 2% FBS (HAdV) or VDB (MS2, *Φ*X174, T4), and were stored at 4°C for no more than six hours prior to enumeration. Each urine and sludge batch was tested in duplicate flasks and triplicate tubes respectively for all organisms. Phage titers were determined in duplicate or triplicate from the same reactor,

whereas HAdV was enumerated once per reactor.

The role of sludge- and urine-specific components in inactivation was investigated in samples of 1:9 diluted urine. Previous to virus spiking, 1:9 diluted urine was processed as follows: to test the role of microbial activity, 1:9 diluted urine samples were filtered at 0.22 µm to remove live microorganisms; to inhibit enzyme activity, samples were first filtered at 0.22 µm to remove microorganisms and the remaining enzymes were heat-inactivated at 65°C for 30 minutes; and to minimize the influence of cations on inactivation, the complexing agent ethylenediaminetetraacetic acid (EDTA; Acros) was added to urine to obtain a final concentration of 10 and 50 mmol L^{-1}.

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

$$ln\frac{C}{C_0} = -k_{obs}t \tag{4.1}$$

where C_0 and C [PFU or MPNCU mL^{-1}] are the virus concentrations at time 0 (initial) and t, and k_{obs} is the first-order inactivation rate constant [day^{-1}]. The data of all replicates were pooled and the 95% confidence interval of k_{obs} was calculated from the standard error of the slope of the pooled data.

4.3 Results and Discussions

Inactivation kinetics. Viruses in all matrices studied exhibited a loss of infectivity with time, and inactivation followed first-order kinetics (Figure 4.1 and see Appendix C, Table C.2). For MS2, which was tested in all matrices, only little variability in k_{obs} was observed between the different batches of raw stored urine (U4-U6, U12-U15) despite the fact that the urine was collected from different sources and in different years. As expected, the ssRNA virus MS2 was inactivated more readily than the DNA viruses, except in S2, where the inactivation of HAdV was fastest. In raw urine at 35°C, a four log_{10} (99.99%) inactivation was achieved within 3 days for MS2, whereas it took more than 100 days to reach the same level of inactivation for T4. In sludge the differences among ssRNA and DNA viruses were slightly smaller with a four log_{10} loss being achieved within 15 and 207 days at 35 °C for MS2 and T4 respectively.

The first-order kinetic behavior indicates that solutions conditions were stable over the course of the inactivation experiments. Correspondingly, a characterization of the composition of stored urine over time showed that both pH and ion concentrations were stable when stored at 20°C or 35°C. For sludge S2, however, the concentration of NH_4^+/NH_3 doubled over the course of 14 days, and the concentrations of Ca^{2+}, Mg^{2+}, SO_4^{2-} decreased two-fold, whereas pH and other ions in solution were more stable. However, the impact of these changes on the solution constituents assumed to govern inactivation (i.e., bases such as NH_3 and HCO_3^-, which are present at significant concentrations and have conjugated acids with a high pK_a;

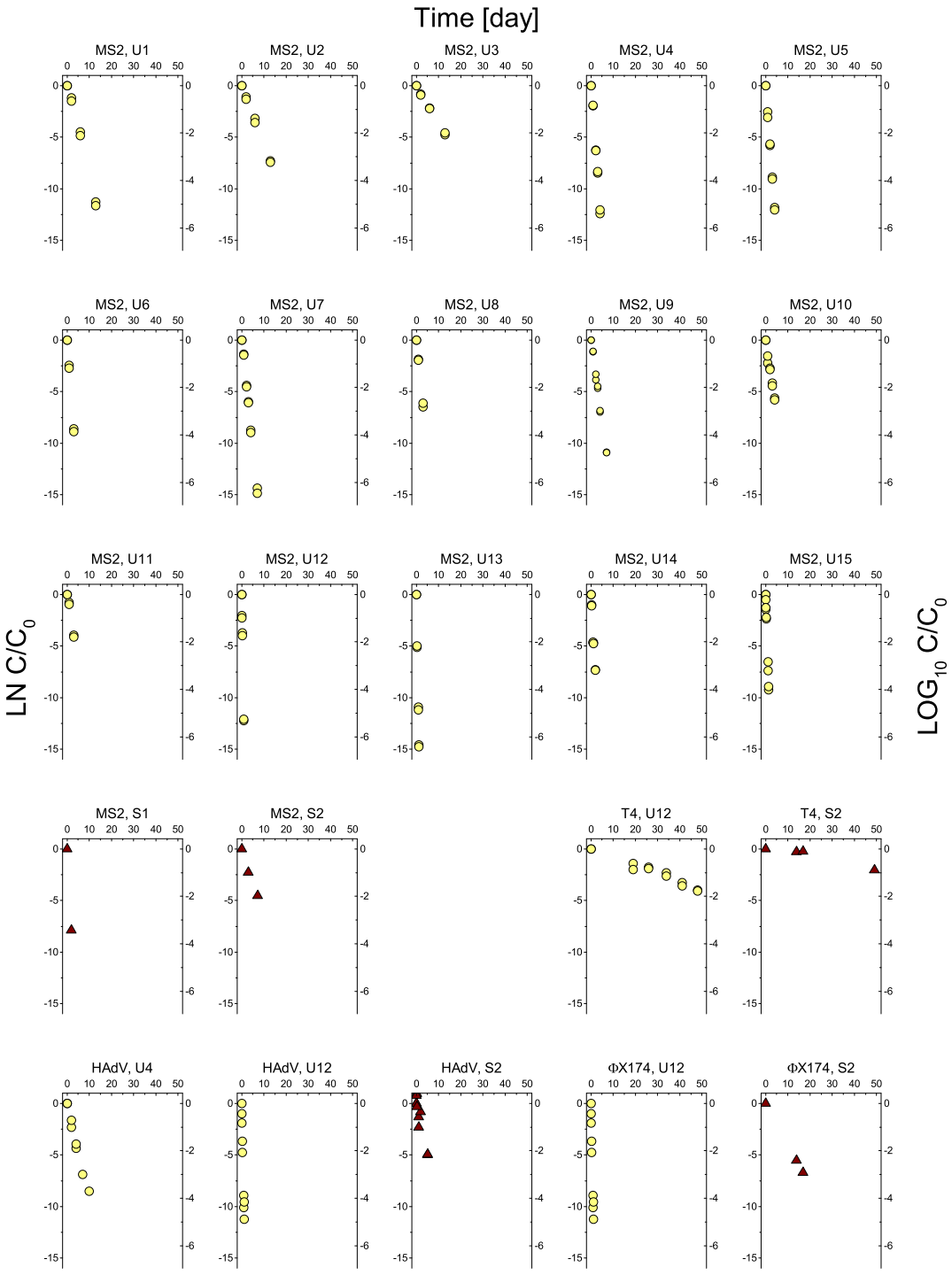


Figure 4.1: *Kinetics of MS2, T4, HAdV and ΦX174 inactivation in stored urine (yellow; U1-U15) and sludge (brown; S1-S2).*

see following section for details) remained small, with an increase from 0.9 to 1.6 mmol L^{-1} and 24.6 to 29.9 mmol L^{-1} for NH_3 and HCO_3^- respectively. For any downstream analysis, the sludge composition was calculated from the average of initial and final for sludge S2, whereas only the composition at initial time was assessed for sludge S1.

The inactivation kinetics observed in this work are consistent with reports by Höglund et al.[97] and Vinneras et al.,[60] who reported first-order inactivation behavior for Salmonella phage 28B and rotavirus in stored urine. Deviation from first-order inactivation was also reported by Vinneras et al. who observed a two phase inactivation behavior for MS2 and ΦX174, with an initial fast inactivation followed by a slow first-order reduction.[60]

Prediction of MS2 inactivation in urine and sludge. MS2 inactivation was tested in 15 urine samples and two different sludge samples, which covered a range of solution conditions and experimental temperatures. This data set was used to challenge the predictive model of MS2 inactivation discussed in Chapter 2, which was established for well-controlled solutions. Specifically, the inactivation rate constants of MS2 in laboratory solutions could be predicted as follows:

$$k_{pred} = \sum_j \{j\} k_j \tag{4.2}$$

Where {j} is the activity in [mol L^{-1}] of bases (or nucleophiles) present in solutions that participate in the base-catalyzed transesterification of ssRNA. k_j [day^{-1} L mol^{-1}], the second order inactivation rate constant associated with the inactivating species j, can be determined according to the Brønsted catalysis law:

$$log_{10} k_j = \beta pK_a + D \tag{4.3}$$

where the parameter β is the Brønsted coefficient, and D is a constant, both specific to MS2. For MS2 inactivation, a β of 0.41 was determined at 35°C with pure solution of different bases(see Chapter 2, Figure 2.9). To predict inactivation at different temperatures, the temperature-dependence of each parameter in the Brønsted catalysis law (eq. 4.3) must be considered. Though the value of β was determined at 35°C, it is not expected to change over the temperature range considered herein. Two pieces of evidence can be cited to support this claim: first, the coefficient β determined experimentally at 20 and 35°C was the same (see Appendix C, Figure C.1); and second, literature reports on a similar reaction, namely the base-catalyzed decomposition of nitramide, showed no relevant temperature dependence of the Brønsted plot slopes for a temperature range 15-45°C.[199,200]

In contrast to β, pK_a, k_j and D are dependent on temperature. For any given temperature, D(T) was estimated based on available experimental data for j=NH_3 as follows:

$$D(T) = log_{10} k_{NH_3}(T) - 0.41 pK_{a,NH_3}(T) \tag{4.4}$$

Where $pK_a(T)$ values are calculated according to Appendix C (see *Determination of pKa (as a function of temperature (T))*, and $k_{NH_3}(T)$ is determined by the Arrhenius relationship reported in Chapter 2, Figure 2.3:

$$k_{NH_3} = exp\left(-5356.5\frac{1}{T} + 22.043\right) \tag{4.5}$$

Finally, it is apparent from equations 4.2 and 4.3 that only those bases contribute to inactivation of MS2 that are present at a significant activity (eq. 4.2), and that have a conjugated acid with a relatively high pKa (eq. 4.3). Given the composition of the matrices used herein, the only bases (j) considered were therefore OH^-, NH_3, CO_3^{2-} HCO_3^-, PO_4^{3-} and HPO_4^{2-}. Their activities were determined as described in Section 4.2.

The correspondence between predicted and observed inactivation rate constants is shown in Figure 4.2. Most of the predictions fell within 70%-140% of k_{obs}. Generally, the model had a tendency to overestimate the observations. This behavior remains unexplained, although it may be linked to the precision of the measured ion concentrations and the PHREEQC estimation of the ion activities. Nevertheless, we can thus conclude that the predictive model established under well-controlled laboratory conditions also applies reasonably well to real matrices.

A brief analysis of the model sensitivity was conducted to assess the influence of pH and temperature, and the inclusion of measured ion concentrations in the model, on the accuracy of the prediction. Specifically, we re-assessed the model prediction for all 17 samples at either pH values of 0.1 units surrounding the measured value or at temperatures of 1°C surrounding the measured temperature. In addition, predictions were carried out that included only NH_4^+/NH_3, or TIC $+NH_4^+/NH_3$, but none of the other ions in solution. This analysis revealed similar sensitivity to shifts in 0.1 pH units as to shifts in 1°C (Figure 4.3). The pH sensitivity may be especially relevant because pH measurement in matrices such as urine or sludge can be difficult to obtain, as the measurement is not always stable. A relatively minor error in the measurement may thus lead to an inaccurate k_{pred}. Interestingly, no relevant differences in the prediction were observed if all ions were taken into account or only the carbonates and ammonium (Figure 4.3). Thus, measurements of temperature, pH, TIC and NH_4^+/NH_3 are sufficient to obtain an accurate prediction of MS2 inactivation. Removal of TIC from the prediction led to a decrease of the prediction accuracy (Figure 4.3). This highlights the importance of carbonate in MS2 inactivation in stored urine or sludge. Carbonate and bicarbonate contributed between 15 and 40% to the total k_{obs} in stored urine (Figure 4.4). The contribution of carbonate species was even higher (> 50%) in sludge S2, which had a pH<8.0 and equivalent amounts of TIC and NH_4^+/NH_3 (Figure 4.4).

The only matrix for which k_{pred} deviated significantly from k_{obs} was 1:9 diluted urine, in which k_{obs} was consistently underestimated by more than 40% (inset in Figure 4.2). This indicates that additional inactivating processes occur in 1:9 diluted urine that are not accounted for in the model. Three possibilities were explored. First, the influence of microbial or enzymatic

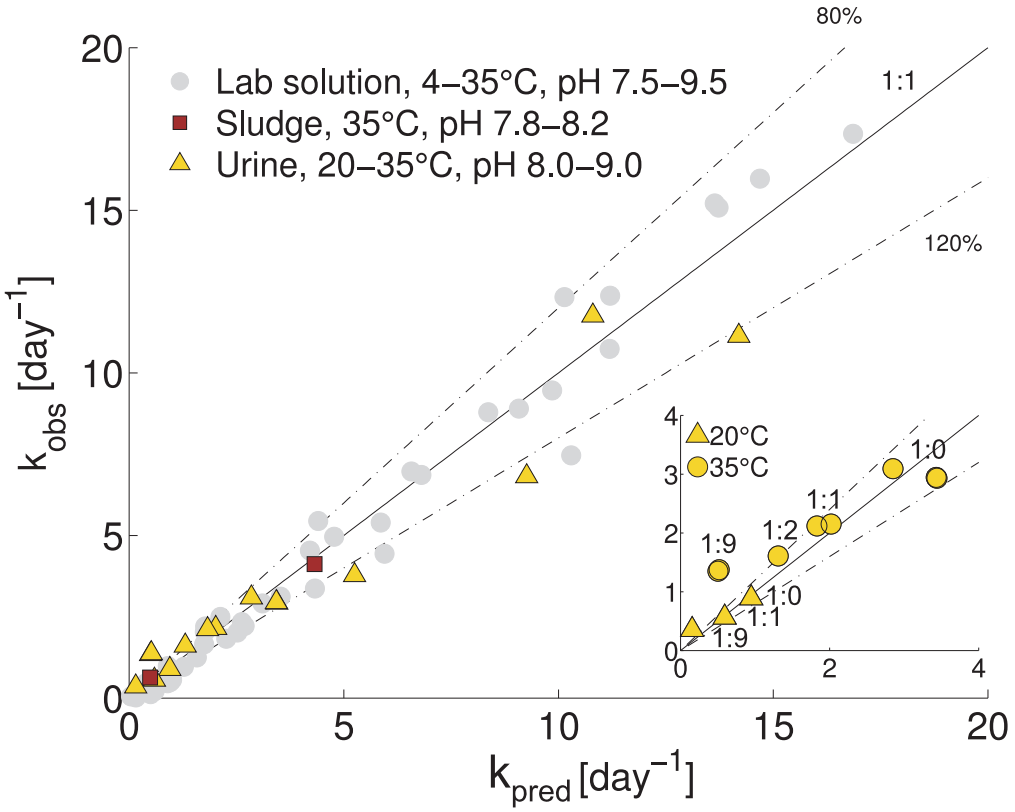


Figure 4.2: *Comparison of measured and predicted MS2 inactivation rate constants for stored urine and sludge. For comparison, data from lab solutions (Chapter 2; grey circles) are also shown. Values of k_{pred} were determined from equations 4.2 and 4.3. The solid line represents a 1:1 relation between measurement and prediction (k_{pred}/k_{obs} = 1). Dashed lines indicate 80% and 120% of k_{pred}/k_{obs} (i.e., k_{pred}/k_{obs} = 0.8 and 1.2 respectively). The inset shows k_{pred} versus k_{obs} for different dilutions of urine. The urine:water ratio for each data point is indicated.*

activity (MEAct) was investigated. The rationale for this test was that dilution of urine may lower the concentration of inhibitory substances present in urine, such that MEAct could be enhanced in diluted urine compared to concentrated urine. However, neither filtration to remove microorganisms nor heating of the solution to inactivate enzymes affect the inactivation kinetics. Hence, MEAct could not account for the differences between k_{obs} and k_{pred} (Figure 4.5, green bars).

Second, the influence of metal ions was assessed. Metal ions are known to promote base-catalyzed RNA transesterification;[131,137] therefore the higher-than-predicted k_{obs} might be explained by the action of metal ions in urine. To eliminate free metal ions from solution, EDTA, a well-known metal complexing agent, was added to the 1:9 diluted urine. This was found to decrease of the k_{obs} to a value close to k_{pred}. Thus, the presence of EDTA suppressed the action of the urine constituents responsible for the higher k_{obs}. Interestingly, if ammonia (as NH_4Cl) was added to (EDTA-free) 1:9 diluted urine, k_{obs} increased as expected based on

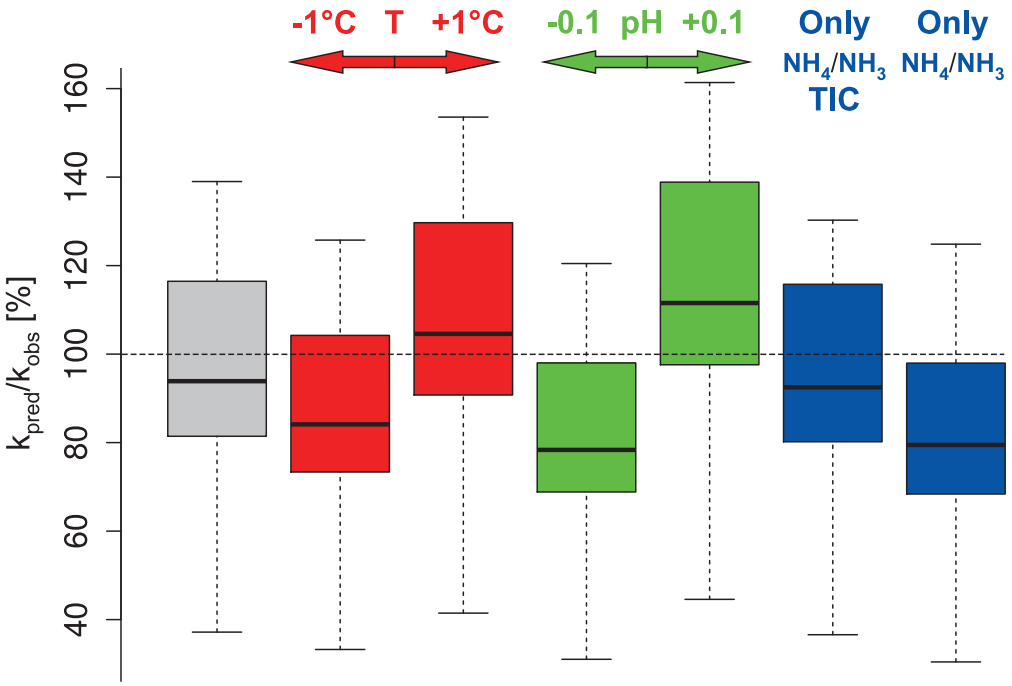


Figure 4.3: *Sensitivity of the model prediction to temperature change (red), pH change (green) and TIC and NH_4^+/NH_3 concentrations (blue) in stored urine and sludge. The prediction corresponding to the measured properties of stored urine and sludge (see Appendix C, Table C.1) is represented in grey. Data are shown in boxplot format, where the thick line indicates the median, the box is bounded by the first and third quartiles and the whiskers indicates the minimum and maximum of the ratio k_{pred}/k_{obs} (in percentage) determined for all 17 samples tested.*

NH_3(aq) (see Appendix C, FigureC.2). The effect of ammonia was thus additive to that of the metal ions, resulting in an overall higher inactivation than in other urine matrices with the same NH_3 activity. We currently cannot explain why urine dilution, followed by ammonia addition results in higher inactivation than an equivalent ammonia activity in undiluted urine.

Finally, underestimation of the true inactivation may also be linked to the model itself which herein only considered the six bases (OH^-, NH_3, CO_3^{2-}, HCO_3^-, PO_4^{3-} and HPO_4^{2-}) determined in stored urine and sludge. However, odorous compound such as sulfide and 4-methylphenol ($CH_3C_6H_4(OH)$), which have high pK_a values (at room temperature, $pK_a(H_2S/HS^-)$ = 6.99, $pK_a(HS^-/S^{2-})$ = 12.92 and $pK_a(CH_3C_6H_4(OH)/CH_3C_6H_4O^-)$ = 10.6) may also contribute to the base-catalyzed inactivation. The concentration of total sulfide and 4-methylphenol were shown to be in the mmol L^{-1} range in stored urine and fecal sludge.[201,202]

In general, the ability of the model to accurately predict MS2 inactivation supports that the

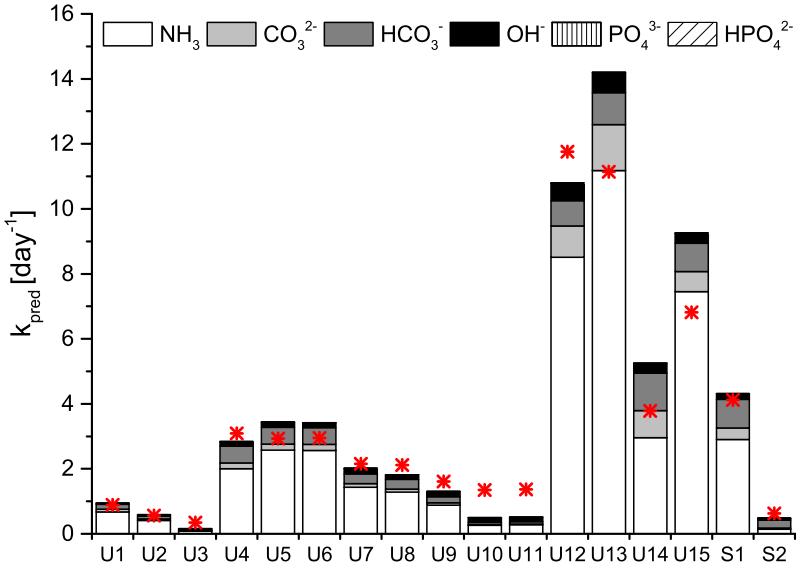


Figure 4.4: *Contribution of the main bases present in stored urine and sludge to the k_{pred} of MS2. k_{obs} is depicted by *.*

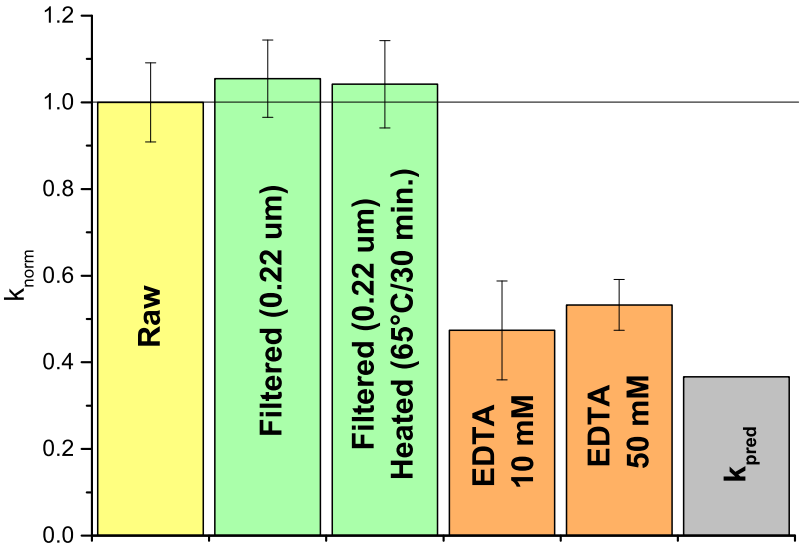


Figure 4.5: *MS2 inactivation rate constants determined at 35°C in 1:9 diluted urine. MS2 inactivation in the absence of microbial or enzymatic activity (green) or metal ions (red) was tested and compared to k_{obs} (yellow) and k_{pred} (grey). k_{norm} corresponds to the ratio of the k_{obs} determined in the filtered, heated or EDTA-containing samples and k_{obs} in raw 1:9 diluted urine (yellow). Error bars depict 95% confidence interval associated with k_{norm}.*

dominant underlying mechanism of inactivation was base-catalyzed transesterification of the ssRNA genome. This appears to be the case in both synthetic solutions and in real matrices such as stored urine and sludge. Consistent with our results, Gao et al.[203] recently reported

that during mesophilic anaerobic treatment of Coxsackievirus (ssRNA), the viral genome was the main inactivating target. Specifically, the virus lost its ability to replicate, whereas the viral proteins remained sufficiently intact to mediate the transfer of the genome into the host cells. To ensure that the model yields a good prediction of MS2 behavior in all type of HEAM, however, more research, especially involving fecal sludge with a lower liquid fraction (typical value: TS between 20-95%[100]), is still needed.

Influence of genome type on virus inactivation kinetics in urine and sludge. In Chapter 3, it was shown that considerable heterogeneity exists among the inactivation kinetics of different viruses. Specifically, the type of viral genome was shown to determine virus fate in laboratory solution mimicking the conditions expected during storage or mesophilic digestion of HEAM. DNA viruses were shown to be consistently more persistent than ssRNA virus. Therefore, to investigate the inactivation of different viruses in real matrices, we focused on comparing the sensitive ssRNA MS2 phage with DNA viruses, specifically phage ΦX174 (ssDNA), phage T4 (dsDNA) and HAdV (dsDNA). Inactivation kinetics were assessed in stored urine and in sludge at 35°C, and were compared to those obtained in laboratory solution (Chapter 3) with and without NH_3(aq), (Figure 4.6).

T4 and ΦX174 showed consistently higher resistance to inactivation than MS2 (Figure 4.6) in all matrices considered. This behavior was expected from the results obtained in laboratory solutions (Chapter 3) and confirmed the higher sensitivity of ssRNA viruses to NH_3(aq) and mildly alkaline pH. Interestingly, however, ΦX174 exhibited a higher removal than expected in sludge, whereas T4 was not affected by matrix type (Figure 4.6). Whether the removal of ΦX174 is due to true inactivation only or removal by adsorption to sludge particles remains to be investigated. Furthermore, even though phage growth is not expected outside the gut and laboratory conditions,[204,205] it ultimately would need to be verified in our matrices.

HAdV, in contrast, revealed a more variable behavior. As observed in laboratory solutions, it was consistently more sensitive than T4 and ΦX174. As expected, HAdV was furthermore more resistant than MS2 in stored urine (U4) at pH 8.2 and approximately 20 mmol L^{-1} {NH_3(aq)} (Figure 4.6). However, in stored urine with a higher pH and NH_3(aq) content (U12) and in sludge, the inactivation of HAdV increased dramatically, resulting in similar to greater inactivation rate constants compared to MS2 (Figure 4.6). The matrix composition (Table 4.1 and see Appendix C, Table C.1) with respect to NH_3 nor pH could not account for these differences. Although increasing {NH_3(aq)} and pH should inhibit microbial activity (see Chapter 3, Introduction), our best hypothesis is that the enhanced inactivation is due to MEAct. This hypothesis, which remains to be tested, is supported by literature data suggesting the susceptibility of HAdV to MEAct: at 37°C, HAdV was found to be inactivated in wastewater at a rate of 13.2 day^{-1} (9.89 day^{-1} in our stored urine at 35°C). This inactivation was independent of the wastewater dilution, and no significant decrease were observed in mineral water for the same temperature.[206]

Besides HAdV, several studies have also shown enterovirus to be sensitive to MEAct.[66–68]

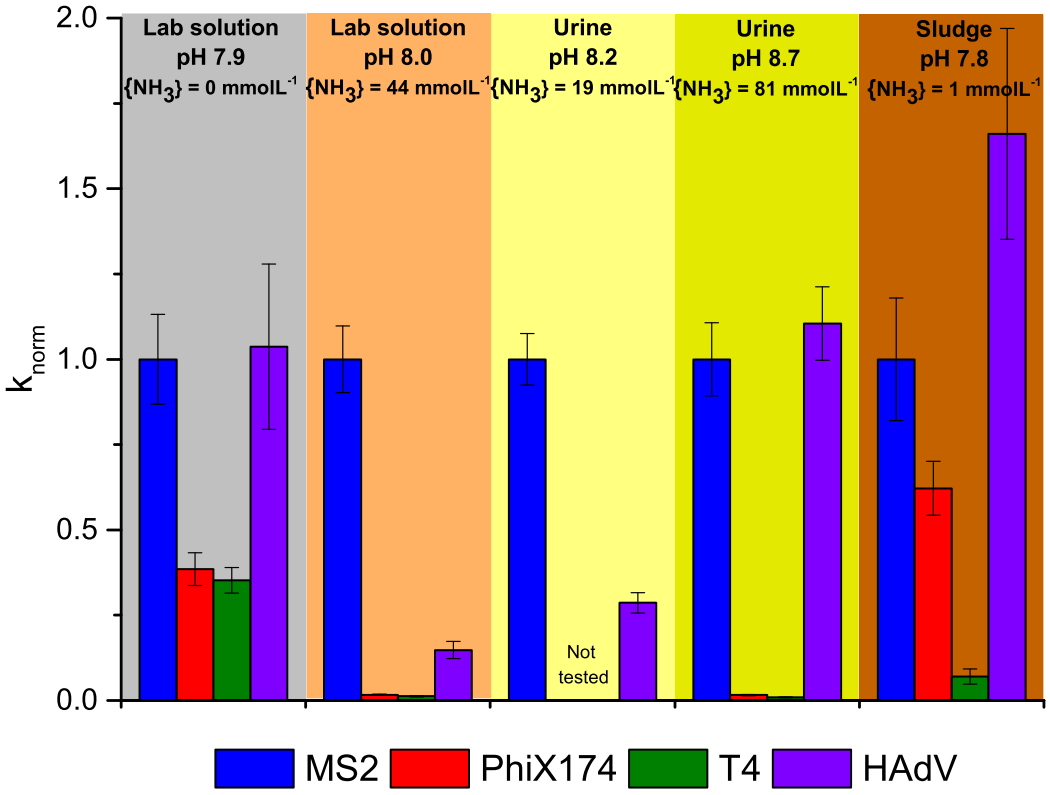


Figure 4.6: *Comparison of virus inactivation in laboratory solution, stored urine (U4, U12) and sludge (S2) at 35°C. To facilitate comparison, measured inactivation rate constants were normalized by that of MS2 for each condition studied (k_{norm}). The absolute rate constants are listed in Appendix C, Table C.2. Error bars depict 95% confidence interval associated with k_{norm}.*

Interestingly, it was observed that viruses exhibited varying degrees of sensitivity to MEAct, with notably phage MS2 and poliovirus being insensitive and coxsackievirus and hepatitis A being sensitive to MEAct.[207] Furthermore, Bischel et al.[208] showed that biological activity of a urine nitrification reactor did not affect phages MS2 and PhiX174 inactivation. The persistence of MS2 in the presence of microorganisms is consistent with the data presented herein that demonstrated that MS2 inactivation was not linked to MEAct (Figure 4.5). Finally, adenovirus, reovirus and enterovirus were found to be more sensitive to inactivation than phage ΦX174 during black soldier fly processing of a sludge mixture where MEAct was probably predominant inactivating parameter.[209] This heterogeneous sensitivity to MEAct is also reflected in our data, with phages not being as affected as HAdV by assumed MEAct.

Implications for the sanitation of HEAM. Among enteric viruses, the vast majority have a ssRNA genome. A good understanding of the factors that promote the inactivation of ssRNA viruses during HEAM treatment is therefore particularly important. The data presented herein demonstrates that for ssRNA viruses, inactivation kinetics and mechanisms established in laboratory solutions appear transferable to real matrices. We furthermore demonstrated that

carbonate, NH_4^+/NH_3, pH and temperature are important drivers of inactivation. To optimize their inactivation of ssRNA viruses, these four parameters should therefore be maximized during treatment. However, similar studies should be extended to additional ssRNA viruses to more conclusively define the optimal treatment conditions. In particular, the role of microorganisms should also be considered, since other ssRNA viruses may be more susceptible to microbial activity than MS2.

While viruses with other genome types are not as common, some enteric viruses with public health relevance have DNA genomes (e.g., adenovirus, polyomavirus). For DNA viruses, kinetic insights developed in controlled solutions may not be as readily transferable to real matrices. The kinetics established in laboratory solutions can therefore only be considered as worst case scenarios for the inactivation of DNA viruses in HEAM.

The complex nature of virus inactivation in real matrices is reflected in the literature, where a large variation in virus inactivation kinetics in excreta is reported. Consequently, results contradicting our data can be found: for example, contrary to our findings, others have shown ΦX174 to be inactivated as fast as MS2 in stored urine[60] and stored fecal sludge.[100] Similarly, ssRNA phage f2 and virus Cosackievirus exhibited similar inactivation than rotavirus (dsRNA) during mesophilic anaerobic digestion of sludge with, however, low pH (7.3).[185] On the other hand, other studies provided observations consistent with our data. For example, F-RNA specific coliphages were shown to be more sensitive than somatic coliphage and dsDNA Salmonella phage 28B during mesophilic digestion of raw sewage sludge[150] and the organic fraction of municipal solid waste.[147] Furthermore, somatic coliphages were found to exhibit low sensitivity to the addition of urea, calcium carbonate and sodium percarbonate used to sanitize composted sewage sludge, which is consistent with our finding that ΦX174 and T4 are not affected by the components of stored urine and sludge.[210]

To reconcile the discrepancies between our results and literature reports, as well as among different literature reports, a better understanding of the mechanisms involved in virus inactivation in HEAM is needed. While we believe that we have made progress toward unraveling the main mechanism involved in the inactivation of ssRNA viruses, those responsible for DNA virus inactivation remain to be determined. In this context, we again emphasize the potentially important contribution of microorganisms on virus inactivation. Determining the virus properties that render it susceptible to microbial predation will be an important next step in understanding inactivation in HEAM.

Finally, our data confirms that somatic coliphage such T4 or ΦX174 are conservative indicators of resistant ssDNA, dsDNA and dsRNA viruses (see Chapter 3). However, it should be noted that they seemed too stable to serve as indicators for the inactivation of the more sensitive HAdV in stored urine and sludge.

5 Conclusions, outlook and implication for HEAM treatment

5.1 What have we learned about virus inactivation in waste? Some take-home messages

A better comprehension of virus fate in HEAM

Viruses are very simply constructed organisms: most fecal-orally transmitted, enteric viruses are composed of a few proteins surrounding a ribo- or deoxyribonucleic acid chain of varying length. Their sensitivity to unfavorable conditions, however, is highly diverse. Even among the small number of phages and viruses investigated herein, dramatic differences in inactivation behavior under a relatively narrow range of external conditions was observed.

Viruses show heterogeneous sensitivity under unfavorable conditions such as those typical of HEAM.

This heterogeneous persistence can result from heterogeneity in both of viral constituents and conditions. With this study, we could determine that genome type was the main component driving the differences in virus inactivation under mildly alkaline conditions (pH 7.0-9.5) and temperature lower than 40°C.

Virus genome type is the main discriminating factor of virus inactivation during storage of HEAM.

Specifically, ssRNA viruses were shown to more sensitive than dsRNA and DNA viruses under conditions typical of HEAM storage and mesophilic digestion. A base-catalyzed ssRNA cleavage was shown to be the rate determining process leading to ssRNA virus inactivation. The cleavage of ssRNA genome is much faster than dsRNA and DNA genome or peptide bond cleavage. This explains the higher sensitivity of ssRNA viruses.

During storage of HEAM, viruses such as adenovirus and rotavirus are more persistent than enterovirus.

When conditions become even more adverse, e.g., by increasing pH and temperature beyond 9.0 and 40°C respectively, other processes start to govern virus inactivation. In particular, microbial activity and protein denaturation are predominant. Human viruses seem to be especially sensitive to microbial activity, whereas phages are not.

Microbial activity and protein denaturation are crucial parameters driving virus inactivation during meso-thermophilic digestion and alkaline stabilization of HEAM respectively.

Figure 5.1 offers a graphical rendition of the processes governing virus inactivation in HEAM. This scheme summarizes the effects of pH and temperature on "viral components" and "virus types" in different conditions encountered during HEAM treatment.

A model to predict indicator ssRNA phage MS2 inactivation during storage and mesophilic digestion.

A predictive model for MS2 inactivation was developed and validated in synthetic solution as well as in real matrices (stored urine and sludge). It was found to offer reliable predictions of MS2 inactivation in conditions expected during storage and mesophilic digestion. A minimum of four parameters were found to be needed for a good prediction: pH, temperature, total ammonium and total inorganic carbon. A user friendly interface was developed and a Shiny application[211] is available open source online (Figure 5.2). This model serves two purposes: first, it seeks to aid other researchers to understand and quantify inactivation in HEAM treatment processes if they use MS2 or similar phages as an indicator. Second, it serves as a template for the development of similar tools to predict inactivation of other viruses in different matrices. However, despite the good agreement between prediction and measurement of MS2 inactivation in the lab, the design of treatment processes and recommendations for treatment time should always be verified at scale.

A prediction tool for MS2 inactivation during storage and mesophilic digestion is available on https://lodecrey.shinyapps.io/MS2inactivation/.

A short comparison of MS2 inactivation kinetics predicted by the model and that reported in literature revealed a large inconsistency between model and measurement (data not shown). This highlights the need for a careful and accurate monitoring of the four parameters named above to obtain an accurate prediction.

Role of indicator viruses to assess virus inactivation in waste.

The heterogeneity among virus inactivation kinetics in HEAM makes it difficult to estimate the inactivation behavior of viruses not explicitly tested in the laboratory or field. Conservative

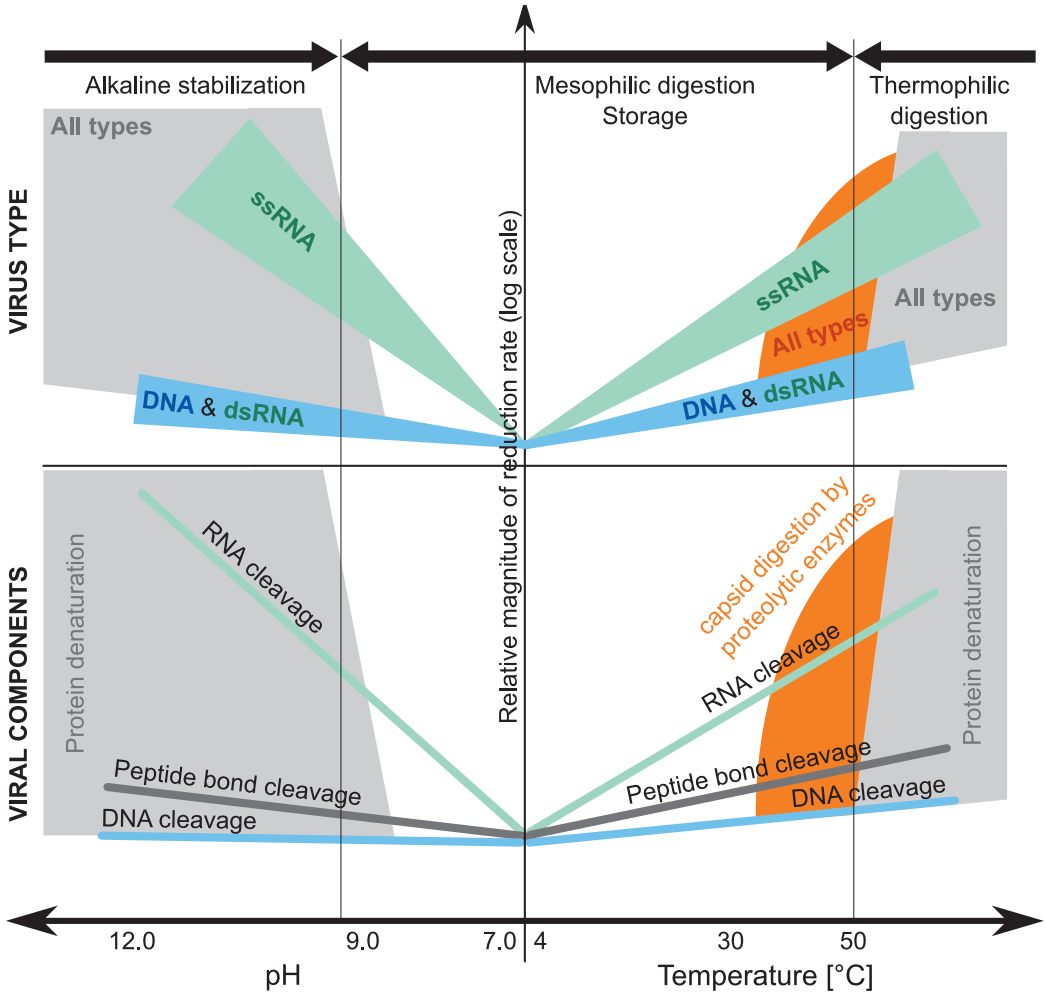


Figure 5.1: *Processes governing virus inactivation in HEAM.*

(or worst-case) indicator viruses are often used in order to make conservative estimates of the treatment time required to reach a desired level of inactivation. Phages represent ideal indicators because they are easily quantifiable and sometimes already present in relevant concentration in HEAM. With this study, we could show that MS2 and T4 would be conservative indicators of ssRNA and dsRNA/DNA enteric viruses, respectively. Unfortunately, as shown in Chapter 3 and 4, they are too conservative in some cases, such as in the presence of active microbial communities. Using those two indicator phages would help to account for virus heterogeneity, but at the same time it would likely lead to overestimation of required treatment times.

MS2 and T4 are very conservative indicators of ssRNA and dsRNA/DNA enteric viruses respectively in HEAM.

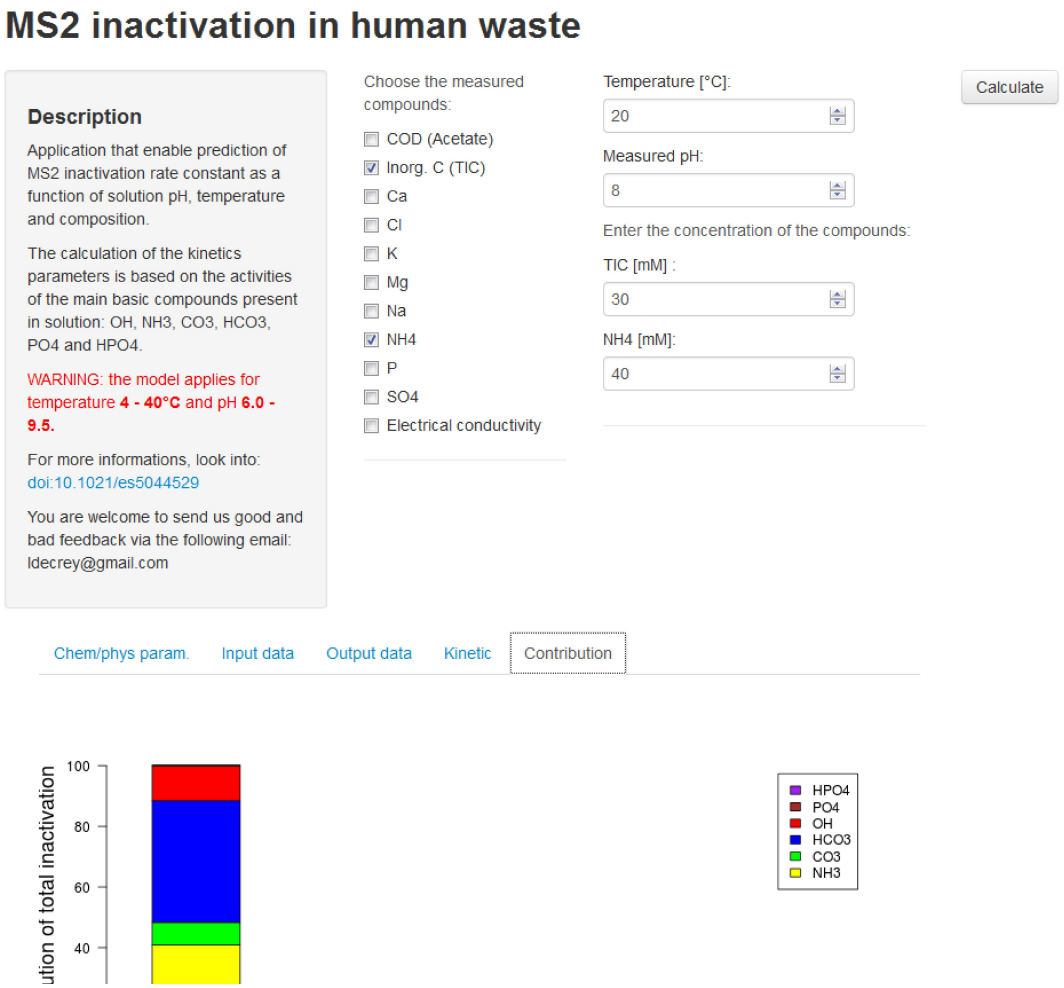


Figure 5.2: *Online application of the MS2 inactivation prediction tool.*

Lessons learned for pathogen management in HEAM.

This work emphasizes the need to strengthen fundamental knowledge on pathogen inactivation, such that the critical parameters driving pathogen inactivation can be identified. An in-depth understanding of the mechanisms underlying inactivation in HEAM constitutes a critical step for predicting inactivation and designing treatment processes. This is exemplified in this study by the finding of different sensitives of viruses with different genome types to inactivation by ammonia: an understanding of the mechanism involved could explain why ssRNA viruses are degraded more readily than dsRNA and DNA viruses. Given this insight, we can now suggest optimal treatment conditions specific to ssRNA viruses or viruses with other genome types. Furthermore, we can conclude that the use of an ssRNA phage as indicator leads to great underestimation of the time required for sanitisation of HEAM during storage.

A study on pathogen inactivation or removal should always seek to the best possible comprehension of the mechanism involved in order to draw pertinent rules that can be applied in the field.

This study furthermore highlights the importance of the method used to report virus infectivity. qPCR based methods are not the way to go in the context of HEAM. This is because inactivation may involve mechanisms involving disruption of the viral proteins, such protein denaturation or digestion by proteolytic enzymes. These processes cannot be captured by qPCR, and qPCR may therefore underestimate virus inactivation. Nevertheless, molecular methods such as qPCR can be useful tools to study genome-based inactivation mechanisms, as illustrated in Chapter 2. Overall, however, infectivity assays they remain the gold standard in determining pathogen infectivity, despite the fact that they are time and resource consuming.

Always seek to use viability or infectivity assays to determine treatment efficiency.

5.2 Future research needs

"As our circle of knowledge expands, so does the circumference of darkness surrounding it." This quotation attributed to Albert Einstein is a commonly accepted paradigm in research. And this study is no exception. The better our comprehension of the mechanism involved in virus inactivation, the more it opens new perspectives on virus inactivation complexity.

Several follow-up questions arise directly from the work performed in this thesis. The greater inactivation of ΦX174 in sludge than in stored urine (Chapter 4), the higher than expected sensitivity of EV to pH 9.0 (Chapter 3), the great sensitivity of HAdV in stored urine and sludge (Chapter 4), the high resistance of ΦX174 to very alkaline pH (Chapter 3) as well as the unexplained disparity amongst inactivation kinetics of DNA viruses under the same conditions (Chapter 3 and 4) remain open questions for future research. In the next steps, special attention should be given to: 1. the influence of microbial and enzyme-related activity on virus inactivation; 2. the virus properties and bulk conditions that promote loss of infectivity by protein denaturation; 3. the role of the solid fraction of sludge in virus protection from inactivation or virus removal from pore water.

To expand on the content of the thesis, the predictive model for MS2 inactivation remains to be tested in sludge with total solid content encountered in fecal sludge i.e., 20-95% of solid fraction. Furthermore, the model could be extended to include human viruses, such as the echovirus studied herein. For this purpose, echovirus inactivation should additionally be examined in stored urine and sludge to test the applicability of the model to human viruses in real matrices. This data would furthermore inform on the role of phages as conservative indicators of human virus inactivation in real matrices. Finally, it would provide additional information on the role of microbial activity in virus inactivation.

More work should also be conducted on characterizing the HEAM matrix. Stored urine may be considered a homogeneous and stable solution when stored in a closed tank. However, the same is not true for feces, urine and ash mixture accumulating in a toilet pit. The temperature, pH and ion content may undergo temporal and spatial variations during storage. The influence of these changing conditions should be considered both in the context of measuring pathogen reduction in HEAM, as well as in establishing model predictions of inactivation.

Finally, the occasional impossibility to achieve complete inactivation of viruses (i.e., zero virus detected) requires some attention. This thesis did not specifically address this problematic, however we can report some observations that may be relevant for future tests. Experiments performed in stored urine typically achieved complete inactivation beyond the detection limit of the virus. In the case of sludge, however, a stable residual fraction of infective viruses was detected, even after prolonged treatment times. Specifically, during thermophilic digestion (55°C) of synthetic sludge, spiked MS2 and T4 revealed a rapid decrease of the phage titer within the first day down to the detection limit.[196] Interestingly, however, we were still able to detect phages around the detection limit of 100 PFU mL^{-1} in samples taken after two and three days. This implies the presence of a resistant virus fraction, and emphasizes the need to understand the mechanisms behind this resistance, and to assess the implications of this small infective fraction for public health.

5.3 Implications for safe management of HEAM

Can viruses be safely managed in HEAM?

The answer to this question depends on the pH and temperature conditions in a HEAM treatment system, as well as the virus type as illustrated in Figure 5.1. Specifically:

- ssRNA viruses such as enterovirus are very susceptible to the conditions encountered in HEAM already at naturally occurring pH and temperature during storage. Especially, bases present in solution enhance inactivation of those viruses through cleavage of their genome. Those viruses can easily be reduced to safe level in HEAM without any tremendous measures. We expect that the same rules hold for all ssRNA viruses potentially transmitted through feces (e.g., Coronavirus (SARS) or Filovirus (Ebola)), though this assumption will have to be verified.
- dsRNA and DNA viruses such as rotavirus, adenovirus and polyomavirus are very resistant to the conditions encountered in HEAM at naturally occurring pH and temperature during storage. Our study however showed that microbial activity has the potential to rapidly reduce adenovirus, but this needs an increase of the temperature close to the optimum of microbial activity. These viruses were furthermore shown to be as sensitive as ssRNA viruses in high alkaline conditions. Therefore, the presence of dsRNA and DNA viruses has to be expected after storage if no special measure is undertaken such as additional heat source or alkalinization with lime.

A decision tree for virus management during storage of human excreta.

Based on the observations made in the different chapters of this thesis, we are able to provide a general and simple decision tree to support the safe management of virus in HEAM during storage and on-site treatment (Figure 5.3). This tool, which is based on the monitoring of conservative indicator (MS2, T4), helps to determine the type of sanitation interventions needed to lower the content of infective viruses in HEAM to a pre-determined level. The tool applies to the treatment of urine, feces, excreta, brown and black water as defined in Tilley et al.[48] and animal manure. A few critical points to be raised include:

1. Choice of the monitored indicator: the conservative indicator, MS2 and T4 suggested here to monitor virus inactivation may lead to overestimation of the time required to achieve a safe end-product as discussed in the section 5.1.
2. Choice of the targeted extent of inactivation (X $\log_{10}$ loss): it has to be defined according to the difference between the expected initial amount of virus and the final level considered to be safe. A common conservative target is 4 $\log_{10}$ loss. The questions remain:
   (a) What initial virus concentration do we expect? One can invest on preliminary test to assess the average concentration of relevant viruses in the raw excreta. But given the spatial and temporal variability of virus shedding (where, when and how often are people infected with viruses), considering the worst case scenario based on existing studies (see Chapter 1) may be the best options to determine the extent to which viruses should be inactivated.
   (b) What is a safe or acceptable level of viruses in HEAM? This remains probably the most open question yet. The answer to this question depends on the fate of the HEAM after storage and treatment. Lower levels of virus are required if HEAM are reused as fertilizer than when HEAM are simply disposed in appropriate dumping site. A better understanding of the risk associated with HEAM reuse or disposal is still needed. Exposure assessments and studies on virus transmission from HEAM could help at getting a better definition of safe product. As long as this definition remains elusive, however, only a product with zero infective viruses can be considered safe.
3. Time constraints: virtually all viruses will end up dying after sufficient storage time. Unfortunately, time is critical, especially because toilet pits fill up and need to be emptied regularly. Therefore, different level of on-site interventions options are given in the decision tree that can reduce the time required to achieve the desire pathogen level.

   The way additives need to be added is not discussed here and is assumed to be homogeneous, even though this issue needs to be addressed in the storage or treatment system of interest. One advantages of sanitation by heat or volatile substances such as NH_3, however, is that a homogeneous distribution can rapidly achieved without mixing as long as the total solids content remains low (<15% in the case of NH_3).[57]

The effect of moisture reduction is not considered herein, but this process may be relevant in the case of fecal sludge storage in UDDT's, or in specific treatments based on drying process. It has been observed that a reduction of moisture content may lead to virus inactivation.[71,72,90,212]

Are further and deeper studies on virus inactivation worth the benefit for HEAM management?

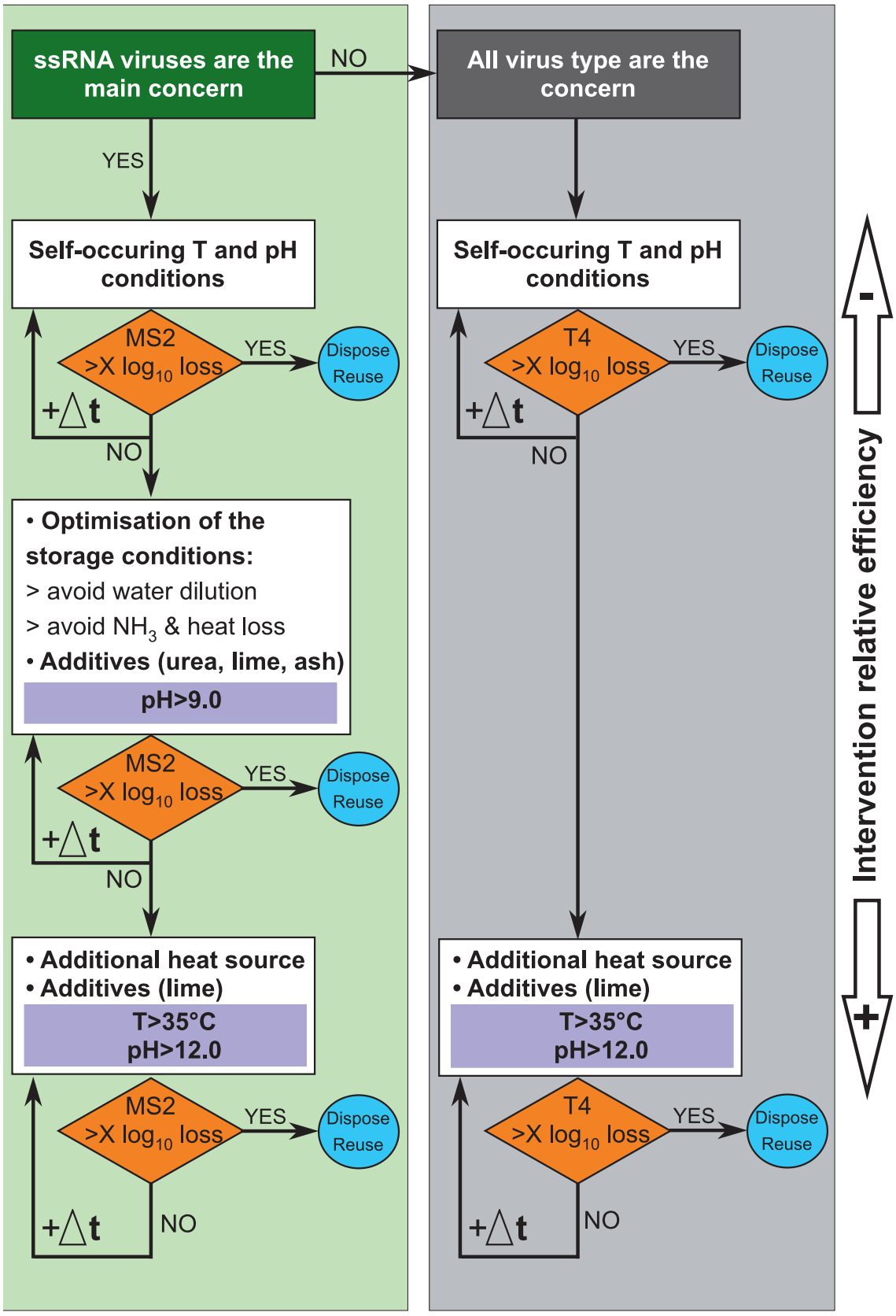
From a scientific point of view, this is no question. Nevertheless, from an engineering point of view, in the context of HEAM management focusing on the single most resistant pathogen might be the best way to go to avoid laborious test and results interpretation. As mentioned in the introduction of this thesis, helminth eggs and viruses were shown to be especially resistant in HEAM during storage, in addition to their low infective dose. My personal experience and readings about pathogen and sanitation during the course of this thesis made me realize that in many cases, even in high pH and temperature treatment process, helminth eggs are actually the most resistant pathogens. Thus, most of the time, treatment processes needs to be design according to the time required to remove helminth eggs. Therefore, in the context of safe HEAM management, I contend that focusing on helminth eggs is probably sufficient to optimize most designs of safe treatment processes.

This personal opinion, however, does not depreciate the importance of this thesis. This personal opinion, however, does not depreciate the importance of this thesis, in particular if the following arguments are considered:

- The results obtained herein are of great relevance, for example, in the context of the transmission of viruses through the fecal-oral route during epidemic outbreak as discussed above and in Chapter 3; the management of animal manure during epizootic events which are mainly related to virus as discussed in the introduction; and in the food industry processing where heat is used to sterilize goods.

- Although helminth eggs are consistently found to be more resistant than viruses, one should also keep in mind that viruses can be excreted in much greater numbers than helminths in HEAM (see Chapter 1). Therefore, viruses may need longer storage or treatment times to reach the targeted extent of inactivation, especially in cases where the difference in inactivation rate constants between viruses and helminth eggs is low (e.g., at elevated temperatures).[213]

- The health risks linked to helminths and viruses are not directly comparable. Virus-related diseases are mostly epidemic and acute, and transmission via excreta should therefore be efficiently controlled. In contrast, helminth-related diseases are usually seen as endemic and chronic, and the sanitation of waste may offer only a small health benefit compared to other measures. Therefore, the design of storage or treatment steps based on helminth egg die-off can lead to an overestimation of the treatment time or


ssRNA viruses are the main concern
NO
All virus type are the concern
YES
Self-occuring T and pH conditions
Self-occuring T and pH conditions
MS2 >X log10 loss
YES
Dispose Reuse
+△t
NO
T4 >X log10 loss
YES
Dispose Reuse
+△t
NO
• Optimisation of the storage conditions:
> avoid water dilution
> avoid NH3 & heat loss
• Additives (urea, lime, ash)
pH>9.0
MS2 >X log10 loss
YES
Dispose Reuse
+△t
NO
• Additional heat source
• Additives (lime)
T>35°C
pH>12.0
• Additional heat source
• Additives (lime)
T>35°C
pH>12.0
MS2 >X log10 loss
YES
Dispose Reuse
+△t
NO
T4 >X log10 loss
YES
Dispose Reuse
+△t
NO
-
Intervention relative efficiency
+

options required to sanitize the excreta and will increase the cost and decrease the ease of implementation in the field without any relevant additional health benefit.

Figure 5.3 *(preceding page)*: *A general decision tree for virus management during HEAM storage. The green scenario relates to cases such as fecal sludge management during outbreaks of viruses, whereas the grey scenario is more generally related to reduction of diarrhea and environmental enteropathy in areas with poor sanitation. Note that at least (X+detection limit) log_{10} number of phages has to be spiked at the beginning of the monitoring process. Virus inactivation due to drying is not considered herein.*

A appendix - Chapter 2

Table A.1: *AmCa and PCa buffer composition and associated k_{obs}.*

T	pH	Na_2CO_3 [mmol L^{-1}]	NH_4Cl [mmol L^{-1}]	NaH_2PO_4*$2H_2O$ [mmol L^{-1}]	HCl [mmol L^{-1}]	NaOH [mmol L^{-1}]	{NH_3(aq)} [mmol L^{-1}]	k_{obs} [day^{-1}]	IC 95%	R^2	k_{pred} [day^{-1}]
4°C	8.68	50.0		50.0		2.8	0.0	0.05	0.01	0.65	0.04
	8.83	50.0	508.0			0.4	23.6	0.46	0.00	0.99	0.39
	8.80	50.0	1497.9			54.4	58.7	0.98	0.03	0.99	0.90
20°C	7.99	50.0		60.0		5.7	0.0	0.02	0.01	0.75	0.15
	8.03	50.0	920.6		26.3		22.6	0.78	0.05	0.99	1.05
	7.95	50.0	1976.8			4.9	38.2	2.50	0.23	0.99	1.70
	9.11	50.0		45.0		0.3	0.0	0.20	0.03	0.97	0.43
	8.86	50.0	194.8		1.9		31.9	1.65	0.12	0.99	1.63
	8.87	50.0	401.7			42.4	63.2	2.91	0.14	0.99	2.95
	8.87	50.0	669.7			88.0	101.0	4.96	0.18	0.99	4.53
	9.58	50.0		35.0		0.4	0.0	0.56	0.19	0.91	0.99
	9.51	50.0	54.0			5.4	26.1	1.82	0.21	0.98	1.97
	9.50	50.0	165.5			49.2	76.3	3.37	0.78	0.95	4.05
28°C	9.03	50.0		44.0		2.2	0.0	0.49	0.07	0.97	0.24
	8.98	50.0	54.8		1.1		18.0	2.20	0.40	0.96	2.07
	8.98	50.0	238.2			42.1	73.2	5.40	0.30	0.99	5.85
	8.99	50.0	455.2			85.4	136.0	7.46	0.32	0.99	10.20
35°C	7.53	50.0		65.0		3.4	0.0	0.12	0.09	0.78	0.47
	7.48	50.0	515.8		42.5		10.7	0.96	0.10	0.98	1.37
	7.43	50.0	895.8		34.7		15.7	2.03	0.02	0.99	1.87
	7.93	50.0		60.0		7.7	0.0	0.21	0.13	0.98	0.56
	8.02	50.0	611.8		5.9		41.1	5.44	0.29	0.99	4.66
	8.04	50.0	1569.9			43.3	101.0	12.33	0.08	0.99	10.90
	8.48	50.0		50.0		3.0	0.0	0.45	0.05	0.94	0.88
	8.41	50.0	53.5		36.8		9.3	1.25	0.11	0.99	1.66
	8.48	50.0	104.8		27.2		20.2	2.33	0.21	0.99	2.86
	8.50	50.0	182.0		8.6		35.5	4.53	0.36	0.99	4.46
	8.49	50.0	342.2			16.1	62.6	6.86	0.43	0.99	7.24
	8.51	50.0	461.9			38.7	85.4	8.90	0.18	0.99	9.62
	8.52	50.0	584.4			60.6	108.0	12.37	0.23	0.99	11.90
	8.53	50.0	729.4			84.4	134.0	15.08	0.18	0.99	14.70
	8.53	50.0	857.3			106.7	155.0	17.35	0.04	0.99	16.90
	9.20	50.0		40.0		0.4	0.0	2.01	0.14	0.99	2.59
	8.98	50.0	24.1		30.4		10.7	2.22	0.09	0.99	2.87
	8.97	50.0	44.6		20.8		19.4	3.12	0.12	0.99	3.74
	9.02	50.0	69.9			0.4	32.2	4.28	0.12	0.99	5.22
	9.03	50.0	223.0			42.9	99.1	10.74	0.24	0.99	12.10
	9.06	50.0	268.6			85.9	123.0	15.21	0.30	0.99	14.60
	9.66	50.0		30.0		2.5	0.0	4.44	0.40	0.99	6.12
	9.60	50.0	17.5		18.8		13.5	6.96	0.90	0.99	7.06
	9.59	50.0	46.6		9.2		35.5	8.79	0.23	0.99	9.16
	9.65	50.0	54.4			12.1	42.8	9.46	0.38	0.99	10.60
	9.63	50.0	128.9			54.1	99.2	15.97	0.31	0.99	15.90

Table A.2: *Composition of the experimental solutions used to determine the effect of bases on MS2, and associated k_{obs}.*

	pH	NH_4Cl [mmol L^{-1}]	HCl [mmol L^{-1}]	NaOH [mmol L^{-1}]	NaCl [mmol L^{-1}]	{NH_3(aq)} [mmol L^{-1}]		k_{obs} [day^{-1}]	IC 95%
NH_3/NH_4^+	8.50	101.9		19.6		21.7		3.39	0.73
	8.50	204.1		37.6		41.0		5.54	0.30
	8.50	318.6		58.1		61.1		8.30	0.57
	8.50	446.3		67.8		82.7		10.77	1.34
						{OH^-} [mmol L^{-1}]			
OH^- (non-buffered)	8.51			5.30E-07	50.0	6.53E-06		0.34	0.13
	9.00			1.69E-06	50.0	2.03E-05		0.80	0.13
	9.20			2.68E-06	50.0	3.18E-05		1.32	0.14
	9.50			5.34E-06	50.0	6.38E-05		2.38	0.42
		Na_2CO_3 [mmol L^{-1}]				{CO_3^{2-}} [mmol L^{-1}]	{HCO_3^-} [mmol L^{-1}]		
HCO_3^-/CO_3^{2-}	8.47	104.9	95.9			0.9	58.2	0.94	0.28
	8.41	544.5	470.7			2.5	176.0	2.45	0.53
	8.50	105.0	94.2			1.0	57.9	0.82	0.14
	8.50	199.8	176.8			1.6	91.5	1.32	0.18
	8.50	393.5	332.9			2.4	141.0	2.14	0.24
	8.50	570.3	483.9			3.0	174.0	2.60	0.23
	9.49	10.0	7.1			1.1	6.3	3.52	0.53
	9.50	22.0	14.5			2.0	11.6	3.29	0.32
	9.51	61.6	40.4			4.1	23.1	4.75	0.32
	9.50	10.0	6.7			1.1	6.2	2.46	0.57
	9.50	61.6	40.2			4.0	23.4	3.60	1.19
	9.50	10.0	7.1			1.1	6.2	4.44	0.87
		Imidazole [mmol L^{-1}]				{Imidazole} [mmol L^{-1}]			
Imidazole	6.99	69.0	28.9			40.0		1.95	0.42
								3.94	0.24
	6.81	140.0	76.8			100.0		3.55	0.29
	6.96	212.0	94.0			120.0		3.63	0.43
	6.98	355.0	162.0			200.0		4.51	0.12
								5.20	0.16
	7.05	532.0	248.6			300.0		6.34	0.33
	6.95	705.0	334.6			400.0		8.07	0.40

B appendix - Chapter 3

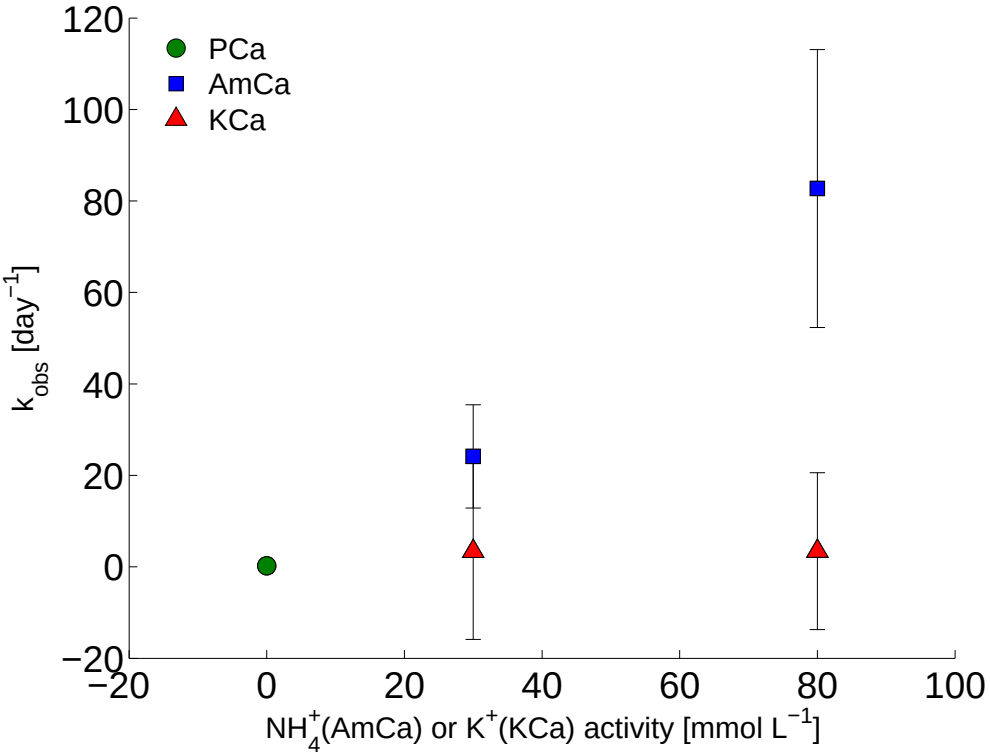


Figure B.1: *Influence of NH_4^+ and K^+ activity on EV k_{obs} at pH 9.0/20°C. The influence of NH_4^+ and K^+ activity were was examined in AmCa and KCa buffer, respectively. The error bars depict the 95% confidence intervals associated with k_{obs}.*

Table B.1: *Composition of working solutions. At pH 9.0/20°C, AmCa 20 and 40 mmol L^{-1} {NH_3(aq)} correspond to 30 and 80 mmol L^{-1} {NH_4^+}respectively and KCa 30 and 80 correspond to 30 and 80 mmol L^{-1} {K^+} respectively.*

T		pH	Na_2CO_3 [mmol L^{-1}]	NH_4Cl [mmol L^{-1}]	NaH_2PO_4*$2H_2O$ [mmol L^{-1}]	HCl [mmol L^{-1}]	NaOH [mmol L^{-1}]	NaCl [mmol L^{-1}]	KCl [mmol L^{-1}]	{NH_3(aq)} [mmol L^{-1}]
20°C	PCa	7.86	50.0		60.0		5.7			0.0
	PCaH	7.87	50.0		60.0		12.0	1770.0		0.0
	AmCa 20	8.03	50.0	920.4		26.3				22.7
	AmCa 40	7.95	50.0	1976.8			4.9			37.6
20°C	PCa	8.86	50.0		45.0		1.0			0.0
	AmCa 20	8.93	50.0	54.9		23.5				10.8
	AmCa 40	8.94	50.0	147.0		1.2				28.4
	KCa 30	9.00	50.0			44.3			44.0	0.0
	KCa 80	9.00	50.0			43.8			113.0	0.0
35°C	PCa	7.92	50.0		60.0		7.0			0.0
	AmCa 20	7.98	50.0	295.1		28.1				19.5
	AmCa 40	8.03	50.0	652.9		5.6				44.3
50°C	PCa	8.00	50.0		60.0		8.8			0.0
60°C	PCa	8.00	50.0		60.0		10.6			0.0
20°C		11.95			10.0		23.7			0.0

Table B.2: *Kinetic parameters associated with phage inactivation: k_{obs} and coefficient of determination (R^2) .*

Phage	pH	T		k_{obs} [day^{-1}]	IC 95%	R^2
MS2	8	20°C	PCa	0.02	0.01	0.75
			PCaH	0.11	0.01	0.98
			AmCa 20	0.78	0.05	0.99
			AmCa 40	2.50	0.23	0.99
	9	20°C	PCa	0.10	0.01	0.99
			AmCa 20	0.86	0.11	0.98
			AmCa 40	1.47	0.14	0.99
	8	35°C	PCa	0.18	0.03	0.98
			AmCa 20	3.18	0.32	0.99
			AmCa 40	5.32	0.58	0.99
	8	50°C	PCa	5.51	0.34	0.99
		60°C	PCa	294.01	42.69	0.97
	12	20°C		$>10^5$		
GA	8	20°C	PCa	0.04	0.01	0.96
			PCaH	0.14	0.01	0.99
			AmCa 20	1.35	0.13	0.99
			AmCa 40	3.33	0.08	0.99
	9	20°C	PCa	0.19	0.02	0.99
			AmCa 20	1.02	0.11	0.98
			AmCa 40	2.30	0.14	0.99
	8	35°C	PCa	0.28	0.04	0.99
			AmCa 20	1.84	0.19	0.99
			AmCa 40	6.10	0.22	0.99
PhiX174	8	20°C	PCa	0.0114	0.0029	0.88
			PCaH	0.0115	0.0012	0.98
			AmCa 20	0.0117	0.0016	0.96
			AmCa 40	0.0110	0.0013	0.97
	9	20°C	PCa	0.0088	0.0034	0.81
			AmCa 20	0.0108	0.0044	0.79
			AmCa 40	0.0124	0.0044	0.84
	8	35°C	PCa	0.0686	0.0094	0.97
			AmCa 20	0.0726	0.0078	0.98
			AmCa 40	0.0893	0.0085	0.99
	8	50°C	PCa	1.1280	0.1548	0.97
		60°C	PCa	265.6200	26.2500	0.99
	12	20°C		0.1024	0.0180	0.93
T4	8	20°C	PCa	0.0104	0.0018	0.93
			PCaH	0.0079	0.0023	0.83
			AmCa 20	0.0106	0.0018	0.93
			AmCa 40	0.0108	0.0014	0.96
	9	20°C	PCa	0.0119	0.0038	0.86
			AmCa 20	0.0263	0.0030	0.98
			AmCa 40	0.0336	0.0021	0.99
	8	35°C	PCa	0.0628	0.0049	0.99
			AmCa 20	0.0560	0.0097	0.97
			AmCa 40	0.0663	0.0118	0.97
	8	50°C	PCa	0.8331	0.0883	0.98
	8	60°C	PCa	196.9300	35.5400	0.95
	12	20°C		$>10^5$		

Table B.3: *Kinetic parameters associated with mammalian virus inactivation: k_{obs} and coefficient of determination (R^2) .*

Virus	pH	T		k_{obs} [day^{-1}]	IC 95%	R^2
HAdV	8	20°C	PCa	0.018	0.008	0.62
			PCaH	0.003	0.007	0.04
			AmCa 20	0.037	0.009	0.71
			AmCa 40	0.060	0.011	0.83
	9	20°C	PCa	0.030	0.019	0.80
			AmCa 20	0.061	0.025	0.91
			AmCa 40	0.111	0.026	0.97
	8	35°C	PCa	0.185	0.056	0.81
			AmCa 20	0.468	0.120	0.90
			AmCa 40	0.786	0.205	0.94
ReoV	8	20°C	PCa	0.040	0.123	0.04
			PCaH	0.048	0.124	0.05
			AmCa 20	0.186	0.103	0.52
			AmCa 40	0.174	0.131	0.37
	9	20°C	PCa	0.015	0.028	0.09
			AmCa 20	0.069	0.031	0.59
			AmCa 40	0.089	0.040	0.58
	8	35°C	PCa	0.154	0.085	0.56
			AmCa 20	0.352	0.041	0.97
			AmCa 40	0.470	0.067	0.96
EV	8	20°C	PCa	0.15	0.03	0.92
			PCaH	0.25	0.06	0.91
			AmCa 20	4.97	1.51	0.84
			AmCa 40	10.24	4.08	0.71
	9	20°C	PCa	0.22	0.15	0.66
			AmCa 20	24.15	11.28	0.75
			AmCa 40	82.72	30.38	0.87
	8	35°C	PCa	3.00	0.87	0.98
			AmCa 20	27.73	13.98	0.79
			AmCa 40	57.78	24.31	0.84

Table B.4: *Kinetic parameters associated with virus/phage inactivation: k_{NH_3} and coefficient of determination (R^2). Data for f2 and poliovirus were obtained from Burge et al.*[93]

Phage/Virus	pH	T	k_{NH_3} [day^{-1}]	IC 95%	R^2
MS2	8	20°C	63.4	38.3	0.89
	9	20°C	46.7	16.6	0.96
	8	35°C	114.8	82.7	0.97
GA	8	20°C	85.2	35.2	0.95
	9	20°C	74.3	1.4	0.99
	8	35°C	133.3	110.8	0.96
PhiX174	8	20°C	-0.009	0.031	0.20
	9	20°C	0.122	0.041	0.96
	8	35°C	0.480	1.690	0.93
T4	8	20°C	0.009	0.001	0.99
	9	20°C	0.729	0.423	0.90
	8	35°C	0.090	0.930	0.16
HAdV	8	20°C	1.1	0.4	0.97
	9	20°C	2.9	0.0	1.00
	8	35°C	13.6	2.1	0.99
ReoV	8	20°C	3.8	3.4	0.79
	9	20°C	2.4	1.8	0.85
	8	35°C	7.0	6.6	0.95
EV	8	20°C	264.0	66.3	0.98
	9	20°C	2944.3	519.7	0.99
	8	35°C	1236.1	72.8	0.99
f2	8	20°C	35.3	5.4	0.99
Poliovirus	8	20°C	235.0	91.3	0.97

Table B.5: *Comparison of DNA, RNA and protein cleavage rate constants.*

	Spontaneous DNA hydrolysis[161]	
A	**Spontaneous hydrolysis of phosphodiester backbone (25°C, pH 7.0)[214]**	
	[s-1]	[day^{-1}]
	1.00E-15	8.64E-11
B	**Hydrolytic deamination of DNA base (37°C, pH 7.4) → mutagenesis, no evidence of cleavage[161]**	
		[day^{-1}]
	dsDNA	3.79E-08
	ssDNA	9.49E-06
C	**Hydrolysis of phosphodiester backbone through abasic site (C1 and C2 are the rate limiting steps)**	
C1.a	*Depurination/depyrimidation (37°C pH 7.4)[162,163] → acid-catalyze reaction*	
		[day^{-1}]
	pyrimidine (CT)	1.30E-07
	purine (AG)	2.59E-06
C1.b	*Depurination (37°C pH 7.1)[166] → acid-catalyze reaction*	
		[day^{-1}]
	Adenine	1.90E-05
	Guanine	2.16E-05
C2	*Self catalyzed (site specific) DNA depurination[164]*	
		[day^{-1}]
	pH7.0, 37°C	1.01E-02
C3	*Chain breakage at abasic site (alkaline-catalyzed reaction)[161,215]*	
		[day^{-1}]
	pH 7.4, 37°C	1.20E-02-1.25E-01
	in 0.1 M NaOH	2.61E+02
	Spontaneous RNA hydrolysis[126]	
		[day^{-1}]
	pH 7.0, 37°C	1.73E-04
	Spontaneous peptide bond hydrolysis[119]	
		[day^{-1}]
	pH 7.0, 37°C	4.02E-06

C appendix - Chapter 4

Determination of pK_a (as a function of temperature (T; in Kelvin))

- For all species (except phosphate) (according to PHREEQC empirical pK_a determination)

$$log_{10}K = A_1 + A_2T + \frac{A_3}{T} + A_4 log_{10}T + \frac{A_5}{T^2} \tag{C.1}$$

With:

	A1	A2	A3	A4	A5
CO_3^{2-}	107.8975	0.03252849	-5151.79	-38.92561	563713.9
HCO_3^-	356.3094	0.0609196	-21834.37	-126.8339	1684915
NH_3	0.6322	-0.001225	-2835.76	0	0
K_w	-283.971	-0.05069842	13323	102.24447	-1119669

And for OH^- (K_a=[OH][H]/[H_2O]=K_w/55.5): $pK_a(T)$=-$\log_{10}(K_w(T)/55.5)$

- For phosphate (Van't Hoff equation):

$$pK_{a_2} = -log_{10}\left(10^{-pK_{a_1}} exp\left(\frac{\Delta H_r}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)\right)\right) \tag{C.2}$$

With ΔH_r at 25°C:

PO_4/HPO_4	-14.7 kJ mol^{-1}
HPO_4/H_2PO_4	-4.2 kJ mol^{-1}

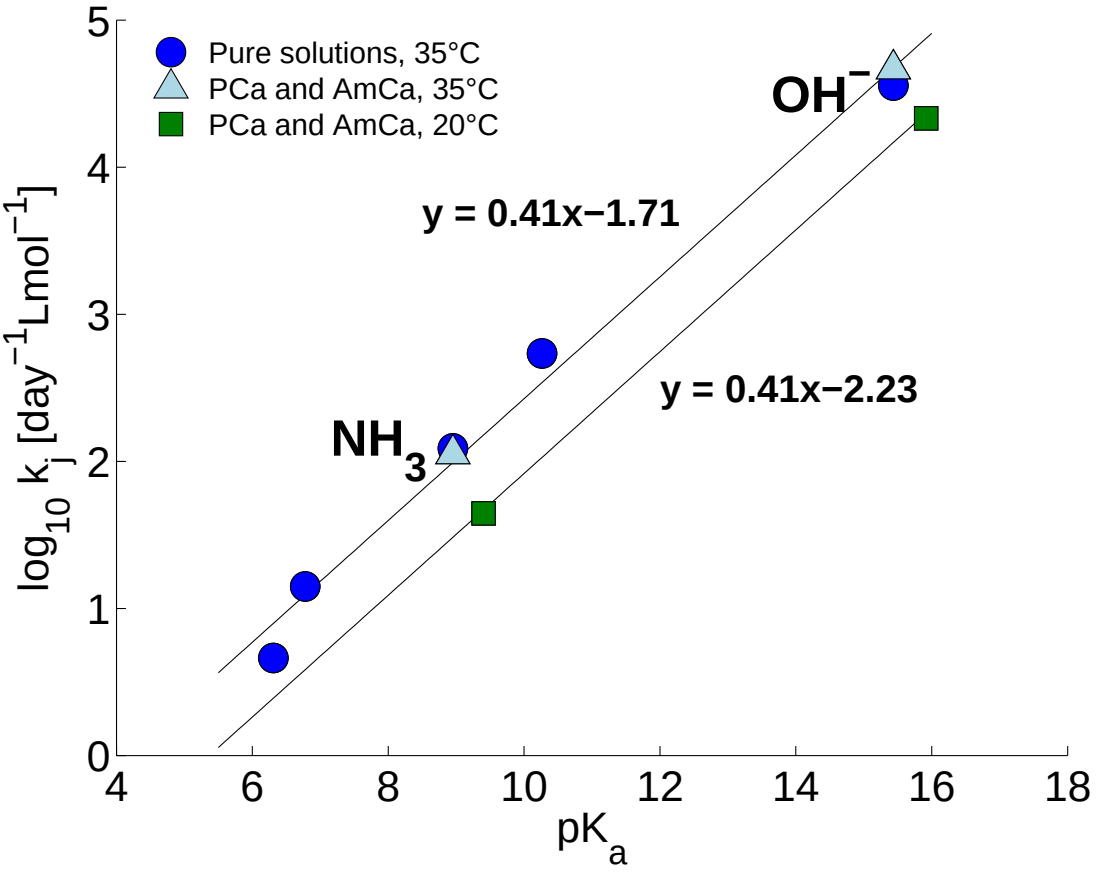


Figure C.1: *Bronsted plot of the pK_a of various bases versus their second-order rate constant for inactivation of MS2. k_j was determined, according to eq. 2.2 , in aqueous solutions of the pure base solution at 35°C (circle)(see Chapter 2) and in PCa and AmCa solution for bases OH^- and NH_3 respectively at 35°C (triangle) and 20°C (square). pK_a values were corrected for temperature (see previous page).*

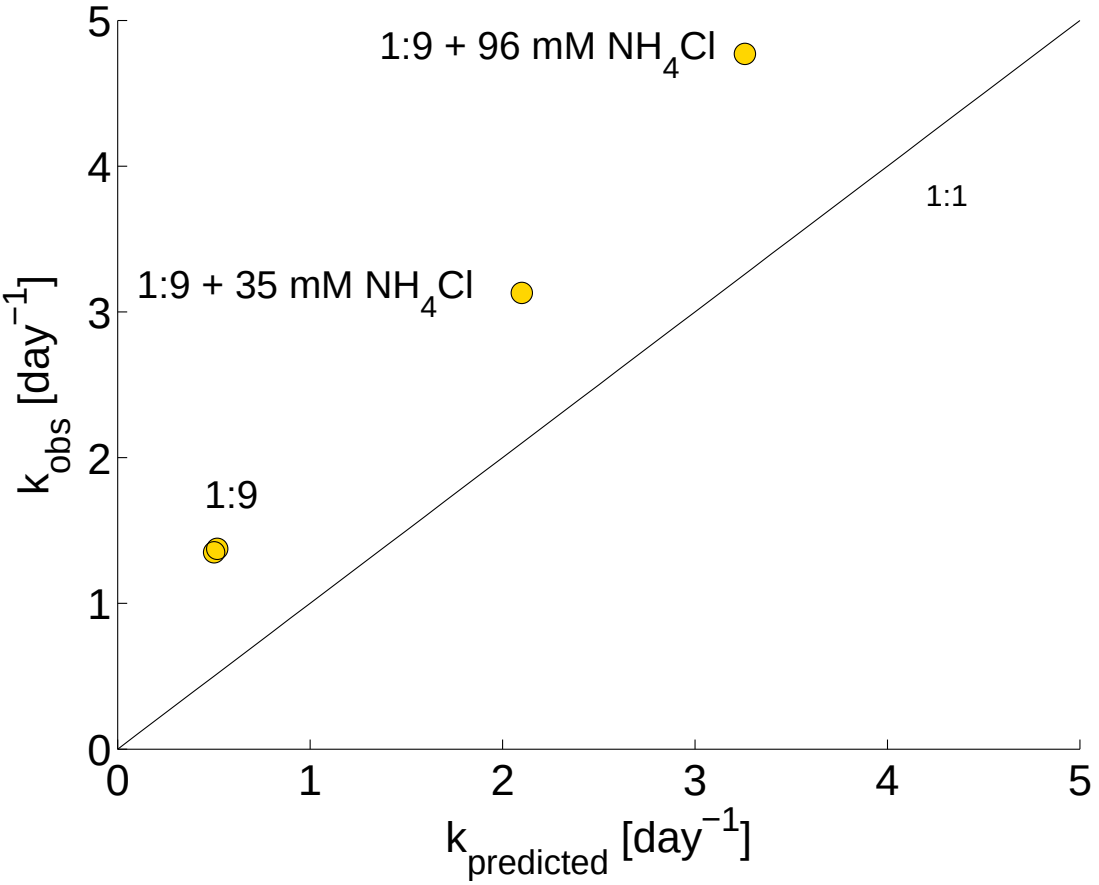


Figure C.2: *Comparison of measured and predicted inactivation rate constants for MS2 at 35°C in 1:9 diluted urine without NH_4Cl (Acros) addition, and with 35 and 96 mmol L^{-1} NH_4Cl. NaOH (9.8 and 22.1 mmol L^{-1}) was added with NH_4Cl to partly compensate for pH drop. Final pH values are: 8.13, 8.52 and 8.46 from lowest to highest ammonia content. The solid line represents the 1:1 ratio between measurement and prediction.*

Table C.1: *Stored urine and sludge characteristics.(Part2)*

ID	Ca		Cl		SCOD		K		Mg		Na		NH_4^+/NH_3		PO_4		TIC		SO_4	
	[mM]	IC 95%	[mM]	IC 95%	[mgO2/L]	IC 95%	[mM]	IC 95%	[mM]	IC 95%	[mM]	IC 95%	[mM]	IC 95%	[mM]	IC 95%	[mM]	IC 95%	[mM]	IC 95%
U1	0.51	0.06	98.2	5.6	5063	278	34.0	1.3	0.05	0.01	84.3	2.8	231.8	23.8	6.3	0.38	109.7	3.7	7.9	0.7
U2	0.25		49.1		2531		17.0		0.03		42.2		123.2	18.1	3.2		54.8		3.9	
U3	0.05		9.8		506		3.4		0.01		8.4		22.7	0.6	0.6		11.0		0.8	
U4	0.51	0.06	98.2	5.6	5063	278	34.0	1.3	0.05	0.01	84.3	2.8	213.9	12.2	6.3	0.38	109.7	3.7	7.9	0.7
U5													254.0	26.3						
U6													252.3	21.6						
U7	0.25		49.1		2531		17.0		0.03		42.2		119.6	23.0	3.2		54.8		3.9	
U8													124.4	3.8						
U9	0.15		29.5		1519		10.2		0.02		25.3		74.3	11.5	1.9		32.9		2.4	
U10	0.05		9.8		506		3.4		0.01		8.4		22.5	2.4	0.6		11.0		0.8	
U11													23.9	0.1						
U12	0.19	0.02	98.7	4.1	4085	54	32.9	0.8	<0.01		69.2	1.9	319.7	10.9	10.8	0.43	208.6	1.1	7.9	0.2
U13	0.18	0.01	89.8	3.1	3635	212	30.0	1.3	<0.01		63.6	1.8	378.2	18.4	0.8	0.02	283.4	68.9	8.2	0.2
U14	0.25	0.01	37.1	19.3	367	64	13.4	0.6	0.02	0.00	24.8	0.8	160.0	19.3	0.3	0.01	263.8	154.1	3.5	0.2
U15	0.21	0.02	118.7	3.3	2004	50	21.1	0.6	0.05	0.06	99.9	1.8	434.7	9.8	11.7	1.85	213.6	7.9	10.6	0.2
S1	1.06	0.95	4.8	3.3	2782	789	4.0	1.2	0.38	0.37	8.2	2.8	245.3	144.8	0.2	0.05	171.1	120.4	0.2	0.0
S2	0.89	0.74	3.8	1.1	438	115	1.4	0.2	0.48	0.40	2.8	0.4	27.8	11.1	2.1	0.08	39.6	5.6	0.1	0.1

Table C.2: *Measured (k_{obs}) and predicted (k_{pred}) kinetics data. MS2 k_{obs} in S1 was determined based on two data points only. Note that for the prediction of S1, Ca^{2+} was not considered in the Phreeqc calculation (no convergence to a solution).*

		k_{obs}			k_{pred}	T sensistivity		pH sensistivity		Ions measured	
Virus		[day^{-1}]	IC 95%	R^2	[day^{-1}]	-1°C [day^{-1}]	+1°C [day^{-1}]	-0.1 [day^{-1}]	+0.1 [day^{-1}]	only TIC & NH_4^+ [day^{-1}]	Only NH_4^+ [day^{-1}]
MS2	U1	0.89	0.06	0.995	0.95	0.84	1.07	0.79	1.14	0.94	0.78
	U2	0.56	0.02	0.998	0.59	0.52	0.67	0.49	0.71	0.58	0.49
	U3	0.35	0.02	0.998	0.16	0.14	0.18	0.13	0.19	0.15	0.13
	U4	3.09	0.25	0.990	2.85	2.55	3.17	2.41	3.38	2.83	2.40
	U5	2.93	0.10	0.998	3.44	3.08	3.84	2.89	4.11	3.42	3.02
	U6	2.95	0.20	0.997	3.43	3.07	3.82	2.87	4.09	3.41	3.00
	U7	2.15	0.11	0.995	2.02	1.81	2.25	1.69	2.40	1.99	1.71
	U8	2.12	0.15	0.997	1.83	1.64	2.04	1.53	2.18	1.80	1.53
	U9	1.61	0.10	0.991	1.31	1.18	1.46	1.10	1.57	1.29	1.10
	U10	1.35	0.14	0.984	0.50	0.45	0.56	0.42	0.60	0.49	0.41
	U11	1.37	0.24	0.982	0.52	0.46	0.58	0.43	0.62	0.51	0.43
	U12	11.76	0.38	0.999	10.80	9.72	11.90	9.19	11.80	10.50	10.00
	U13	11.14	1.15	0.989	14.20	12.80	15.60	12.20	16.40	13.40	13.00
	U14	3.78	0.45	0.978	5.25	4.75	5.80	4.55	6.06	4.65	3.70
	U15	6.82	0.46	0.991	9.26	8.31	10.30	7.77	11.00	8.88	8.51
	S1	4.12			4.32	3.88	4.80		5.08	3.83	3.25
	S2	0.63	0.19	0.995	0.49	0.44	0.54	0.44	0.55	0.45	0.21
HAdV	U4	0.89	0.12	0.982							
	U12	9.89	1.14	0.973							
	S2	1.05	0.28	0.884							
T4	U12	0.08	0.01	0.964							
	S2	0.04	0.03	0.941							
PhiX174	U12	0.14	0.02	0.957							
	S2	0.39	0.00	1.000							

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Education

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Intern Supervised by Elisabeth Tilley and Kai M. Udert
Eawag, departement of water and sanitation in developing countries (SANDEC)
Dübendorf, Switzerland
2010, 8 months

Personal Interests

- **Sports:** volleyball (several years at national level), ski/snowboard touring, mountain biking, climbing/bouldering, windsurf/surf, badminton.
- **Arts:** juggling, tapdance, photography, drums.
- **Adventures:** by bike or by foot.

Publications

1. **Decrey, L.**, Kazama, S., Udert, K.M. and Kohn, T. Ammonia as an in-situ sanitizer: inactivation kinetics and mechanisms of the ssRNA virus MS2 by NH3. *Environmental Science and Technology*, **2015**.
2. Pecson, B., **Decrey, L.** and Kohn, T. Photoinactivation of virus on iron-oxide coated sand: enhancing inactivation in sunlit waters. *Water Research*, **2012**.
3. **Decrey, L.**, Udert, K.M., Tilley, E., Pecson, B. and Kohn, T. Fate of the pathogen indicators phage ΦX174 and Ascaris suum eggs during the production of struvite fertilizer from source-separated urine. *Water Research*, **2011**.

Conference presentations

3rd International IWA fecal sludge management conference (FSM3)
Hanoi, Vietnam
January 15-20, 2015

Virus inactivation in human or animal waste. *Decrey, L., Kazama, S., Udert, K.M., and Kohn, T.* Oral presentation. Available on: http://www.susana.org/en/resources/conference-materials-2/2015/259-fsm3.

17th IWA symposium on health-related water microbiology
Florianopolis, Brazil
September 15-20, 2013

Virus inactivation in human waste: uncharged ammonia as an in-situ sanitizer? *Decrey, L., Kazama, S., Udert, K.M., and Kohn, T.* Poster presentation.

3rd Food and environmental virology conference
Lisboa, Portugal
October 7-10, 2012

Virucidal properties of uncharged ammonia. *Decrey, L., Kazama, S., Udert, K.M., and Kohn, T.* Poster presentation.

References

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