Virus Inactivation - Evaluation of Processes used in Biowaste Management

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Abstract

Animal by-products (ABP) Category 3 includes hatchery waste, and also slaughterhouse waste and manure, aimed for use in biogas production. In order to be used as fertilisers, they must be sanitised to reduce pathogenic microorganisms. The initial European regulations regarding processing standards for ABP intended for use in e.g. biogas plants (EC No. 1774/2002) stipulated thermal treatment at 70°C for 60 min. A subsequent amendment (EC No. 208/2006) stated that a validated treatment process may be used if it can show a reduction in thermo-resistant viruses of at least 3 $\log_{10}$, whenever deamed a risk.

Thermal treatments of biogas substrate at 70°C and at 55°C, using porcine parvovirus and swine vesicular disease virus, were performed. As a chemical sanitisation process regarding hatchery waste, ammonia inactivation was tested using the highly pathogenic avian influenza virus H7N1 and the low pathogenic avian influenza virus H5N3. Models for other avian pathogenic ssRNA viruses were bovine parainfluenza virus 3, feline calicivirus and feline coronavirus. As possible indicators for monitoring virus inactivation, bacteriophages MS2, ϕX174, and 28b were evaluated.

Thermal treatment at 70°C for 60 min inactivated porcine parvovirus by 2.6 $\log_{10}$, while phage 28b was unaffected. Swine vesicular disease virus was undetectable after 30 min. Using too thermostable viruses as models for sufficient virus reduction in thermal treatments regarding Category 3 ABP materials and manure intended for biogas or composting plants, according to EU regulations, would make demands on other time-temperature combinations very strict.

All viruses tested were efficiently inactivated by ammonia treatment in hatchery waste, while phage ϕX174 proved too conservative to be used as indicator. Using phage MS2 as a stable indicator to monitor a 3 $\log_{10}$ reduction of ssRNA virus showed that addition of 0.5% w/w ammonia is required, followed by storage for at least 31 h at ≥14°C. In case of an outbreak of e.g. avian influenza, storage for two days at the same conditions was estimated.

Keywords: ABP Category 3, AIV, ammonia, bacteriophage MS2, PPV, SVDV, thermal treatment, virus inactivation

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Sahlström, L., Bagge, E., Emmoth, E., Holmqvist, A., Danielsson-Tham, M.-L., and Albihn, A. (2008). A laboratory study of survival of selected microorganisms after heat treatment of biowaste used in biogas plants. *Bioresource Technology* **99**(16)**,** 7859-7865.

II. Eva Emmoth, Jakob Ottoson, Ann Albihn, Sándor Belák, Björn Vinnerås. Ammonia disinfection of hatchery waste for elimination of single-stranded RNA viruses. (Submitted to Applied and Environmental Microbiology)

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The contribution of Eva Emmoth to the papers included in this thesis was as follows:

I Virus part: 75% planning, 50% analysis and summary of results, 100% performance of work, participation in writing materials and methods, references to introduction and discussion

II 75% planning, 100% performance of work, 50% analysis and summary of results with Ottoson, writing of manuscript with Ottoson, with revision by co-authors

Abbreviations

ABP	Animal by-products
AHEV	Avian hepatitis E virus
ATCC	American Type Culture Collection
BPIV 3	Bovine parainfluenza virus 3
CCM	Cell culture medium
FCV	Feline calicivirus
FCoV	Feline coronavirus
HPAIV	Highly pathogenic avian influenza virus
HW	Hatchery waste
IBV	Infectious bronchitis virus
LPAIV	Low pathogenic avian influenza virus
MS2	*Enterobacteria* phage MS2
NDV	Newcastle disease virus
NH_3	Ammonia
PFU	Plaque-forming units
PPV	Porcine parvovirus
SVDV	Swine vesicular disease virus
$TCID_{50}$	Tissue Culture Infectious $Dose_{50}$
ϕX174	*Enterobacteria* phage ϕX174
28B	*Salmonella* Typhimurium phage 28B

Definitions

D-value	Time required to reduce the number of organisms by 1 $\log_{10}$ (90%)
Substrate	Liquid biowaste from a biogas plant used in Paper I, consisting of Category 3 ABP materials, e.g. manure, blood, fat, together with waste from food industries and biological waste from households, separated at source
Hatchery waste (HW)	Biowaste from a hatchery, used in Paper II, consisting of eggshells and tissue from developing embryos, with a dry matter content of about 60%

Introduction

In society today, a great amount of biological waste (biowaste) is produced, including animal by-products (ABP) such as livestock manure, slaughterhouse waste and hatchery waste (HW). This biowaste contains valuable nutrients, and it is thus desirable for it to be recycled into the food chain by application onto arable land. However, due to the risk of pathogenic microorganisms such as viruses being present, sanitisation of the biowaste prior to use as a fertiliser or soil conditioner is required to prevent pathogen transmission to animals and man.

Biowaste treatment practices include anaerobic digestion in biogas plants, thermal and biological treatments such as composting, and disinfection using chemicals such as ammonia. As the high eggshell content of HW could make it unsuitable for composting and anaerobic digestion, liming is often used in Sweden as a chemical method for sanitation. However, this treatment results in a poor working environment and is technically complicated, due to the high pH (>12) and formation of carbonate and phosphate sediments. As the global production of poultry meat is increasing rapidly, mainly in the form of large-scale production units with dense poultry populations, it is important to find hygienically appropriate disposal methods for poultry waste such as HW. In the event of an epizootic outbreak, such as avian influenza or Newcastle disease, hatcheries could be at risk of virus introduction through e.g. virus-contaminated eggs, egg trays, lorries, or other tools of virus transmission. When an outbreak occurs, special rules for decontamination apply and harsher methods are often used to disinfect all infectious materials.

Strict regulations have been developed for handling ABP, e.g. EC No. 1774/2002 (EU, 2002). This regulation is central to achieving food safety and animal health, as it sets rules to minimise the risk of pathogenic

contaminants and improve the traceability of ABP. It divides ABP into different risk categories, depending on the expected degree of pathogenic contamination, and sets criteria for treatments for each category. As a minimum requirement, Category 3 ABP waste intended for transformation in a biogas or composting plant must be submitted to thermal treatment at 70°C for 60 min (EU, 2002), or an alternative treatment according to amendment EC No. 208/2006 (EU, 2006) that is capable of achieving a reduction of 3 $\log_{10}$ in a thermo-resistant virus, whenever there is a relevant risk.

In the context of the amended EU regulations on ABP, this thesis investigated thermal treatments of biogas substrate regarding inactivation of porcine parvovirus and swine vesicular disease virus. Using HW, we also tested ammonia treatment as a method for inactivation of avian influenza virus and models for Newcastle disease virus, avian infectious bronchitis virus and avian hepatitis E virus, both regarding regular biowaste management and treatment in the event of an epizootic outbreak. In addition, in both the thermal and the ammonia treatments bacteriophages were evaluated or used as indicators of virus inactivation, in order to develop cheap and safe evaluation systems for present and future treatment methods.

Objectives of this thesis

- The overall objective of this thesis was to minimise the risk of transmission of pathogenic viruses to food-producing animals from the handling of animal by-products.

Specific objectives were:

- To assess the virus-inactivating effect of thermal treatments on Category 3 animal by-products intended for biogas or composting plants, in accordance with current EC regulations.
- To investigate the efficiency of ammonia treatment as a disinfection method for hatchery waste in the event of an epizootic outbreak of avian influenza or Newcastle disease, and to devise a routine method for viral sanitation of hatchery waste in accordance with existing animal by-product regulations.
- To investigate the possibility of using bacteriophages as indicators of virus reduction in the biowaste management processes studied.

Background

Viral contamination of biowaste

Biowaste that can be used in biogas or composting plants includes Category 3 ABP materials such as manure, blood and fat. This material should be free of pathogenic viruses, but cross-contamination or other residual infectivity can be a problem (Böhm, 2005). Moreover, subclinically infected animals can result in virus-infected slaughterhouse waste. Enteroviruses are prevalent in pig manure (Derbyshire *et al.*, 1986), with titres of up to 5.7 $\log_{10} g^{-1}$ for swine vesicular disease virus (Sellers, 1982). This is also the virus group that is becoming one of the most problematic due to its gastrointestinal infection route, shedding in faeces and great stability (Ley *et al.*, 2002). Regarding viruses present in HW, ssRNA viruses were tested here, but additional viruses having vertical spread (could be present in the egg) include avian encephalomyelitis-like virus, a tentative hepatovirus (Picornaviridae); inclusion body hepatitis virus, an aviadenovirus; avian leucosis sarcoma virus, a retrovirus; and chicken anaemia virus, a gyrovirus (SVA, 2010). Table 1 shows some characteristics of excretion of the viruses used in these studies, which can be expected to be present in ABP, as well as the viruses for which they acted as models.

Table 1. *Some characteristics of excretion of viruses used in the present studies, which can be expected to be present in ABP, and the viruses for which they acted as models*

Virus	Faeces	Organ	Egg	Reference
Porcine parvovirus	+++	Uterus, foetus	NA	(Mengeling, 1989)
Swine vesicular disease virus	++	Secretions, lesions	NA	(Hedger & Mann, 1989)
High pathogenicity avian influenza	++ up to 4.5 $\log_{10}$ EID_{50} g^{-1}	Resp. secretions up to 7.7 $\log_{10}$ EID_{50} mL^{-1}	Yes	(Spickler *et al.*, 2008) (Swayne & Halvorson, 2008)
Low pathogenicity avian influenza	+ up to 4.3 EID_{50} mL^{-1}	Resp. secretions up to 5.5 $\log_{10}$ EID_{50} mL^{-1}	Yes	(Cappucci *et al.*, 1985) (Swayne & Halvorson, 2008)
Bovine parainfluenza virus 3	(+)	Respiratory secretions 6-7 $\log_{10}$ $TCID_{50}$ mL^{-1}	NA	(Bryson, 1990)
Newcastle disease virus	+++	Intestines, trachea	Yes	(Alexander & Senne, 2008) (Capua *et al.*, 1993) (Chen & Wang, 2002)
Avian hepatitis E virus[a]	+++	Liver, intestines	Yes	(Meng *et al.*, 2008; Guo *et al.*, 2007)
Infectious bronchitis virus[b]	++	Trachea, lungs, kidney	No	(Cavanagh & Gelb Jr, 2008)

NA = not applicable; [ab]models are feline calicivirus and feline coronavirus, not expected in ABP; +++ = primarily excreted in faces; ++ = clearly excreted in faeces; + = lower incidence; (+) = unlikely to be present in faeces. Adapted from (Strauch, 1991).

Zoonotic and potentially zoonotic viruses

Viral zoonoses of concern regarding large-scale animal farms include hepatitis E and influenza A (USEPA, 2005). Table 2 shows some zoonotic or potentially zoonotic viruses of the types studied here, e.g. highly pathogenic avian influenza (HPAIV), with the most probable transmission at close range through bird secretions to human respiratory mucosa (Brankston, 2007). For HPAIV, concerns also exist about gastrointestinal infection through contaminated foods (Vong, 2008). If zoonotic influenza contaminates groundwater it could pose a risk for humans (WHO, 2006).

Swine hepatitis E virus is also considered a zoonotic agent (Bihl *et al.*, 2010; Widén *et al.*, 2010). It has been found in manure (McCreary *et al.*, 2008; Kasorndorkbua *et al.*, 2005) and could also be present in swine liver (Leblanc *et al.*, 2010). Handling ABP waste, e.g. slaughterhouse work handling swine livers, could be a source of zoonotic hepatitis E virus spread

(Perez-Gracia *et al.*, 2007). Moreover, the calicivirus group (here we studied feline calicivirus) is the most common of the food-borne viruses and there is a risk of new emerging strains through recombination (Baert *et al.*, 2010). In contrast, SVDV has evolved from human Coxsackie virus Type 5, and it has retained its affinity for the human coxsackie/adeno receptor (Jimenez-Clavero *et al.*, 2005), but it has lost its zoonotic potential compared with old virus isolates, which mediate cell destruction of human cells.

Table 2. Zoonotic and potentially zoonotic viruses of the types studied, which may be present in ABP

Virus family/genus	Reference	Virus
Orthomyxoviridae	(De Benedictis *et al.*, 2007; Chan, 2002)	Highly pathogenic influenza virus H5*, H7*
	(Smith *et al.*, 2009)	'Novel' swine influenza virus H1N1*
Picornaviridae	(Warren, 1965)	Encephalomyocarditis virus**
	(Brewer *et al.*, 2001)	
Caliciviridae	(Ike *et al.*, 2007)	(Bovine norovirus)**
	(Poel *et al.*, 2000)	(Swine norovirus)**
	(Martella *et al.*, 2008)	(Porcine enteric sapovirus)**
Hepevirus	(Meng, 2010)	Swine hepatitis E virus*

*zoonotic **potentially zoon; ( ) = tentative member of species

EU regulations regarding animal by-products

To minimise the risk of pathogen spread to animals and humans, European Union regulation EC No. 1774/2002 (EU, 2002) lays down health rules concerning animal by-products not intended for human consumption. The EC regulation divides ABP into three categories, depending on the suspected degree of pathogens present.

Category 1 includes ABP from animals with confirmed or suspected transmissible spongiform encephalopathy (TSE), as well as specified risk materials, i.e. carcass parts of bovines or caprines where TSE is at risk but not confirmed, e.g. brain and spinal cord from animals >12 months of age, and intestines from all ruminants. This material must be disposed of by incineration.

Category 2 comprises animals that have died by means other than slaughter, including animals killed in eradication of an epizootic disease, and digestive tract contents and manure for commercial purposes. This material

must be sterilised at 133°C for 20 min at 3 bar, or equivalent heat treatment.

Category 3 material includes parts of slaughtered animals fit for human consumption, hatchery waste, catering waste other than from means of transport operating internationally, and manure intended for e.g. biogas or composting plants. This material must be heated to 70°C for 60 min, or equivalent treatment according to amendment EC No. 208/2006 (EU, 2006). This amendment concerns processing standards for biogas and composting plants, and states that a thermal or a chemical process could be used for Category 3 materials, provided it gives a reduction of at least 3 $\log_{10}$ of a thermo-resistant virus, whenever it is deemed a risk. Alternatively, endogenous indicator organisms may be monitored during the process, provided they are consistently present in the raw material, have approximately the same resistance to the inactivating capacity of the treatment process and are easy to quantify and confirm. Internationally, different rules exist regarding ABP in different countries.

Virus inactivation methods used in biowaste management

Thermal treatments

Many investigations have been carried out regarding thermal treatments in biowaste materials (Table 3). Thermal treatment to reduce viruses in biowaste can involve applying heat through different means, by aerobic digestion or composting, where heat is produced by microorganisms, or by anaerobic digestion, where heat usually has to be added to get a virus-inactivating effect. Any biosolids present can even retain heat and result in faster virus inactivation (Turner & Burton, 1997). An exception is when the virus is embedded in tissue, as this has been shown to protect viruses. For example, foot-and-mouth-disease virus has been shown to maintain its infectivity for 4 h at 85°C (Dimopoullos *et al.*, 1959), while for classical swine fever virus in tissues the rate of inactivation at temperatures above 35°C diminished about 8-fold compared with the same virus in manure (Frøkjær Jensen & Have, 1995).

The mechanism for heat inactivation of naked ssDNA viruses of the Parvovirinae subfamily has been shown to be destruction of the virus capsid, thereby rendering the DNA accessible to degrading enzymes (Blumel *et al.*, 2002), suggesting that different capsid structures influence the susceptibility to heat, the genus *Parvovirus* being one of the most stable. Regarding enteroviruses, heat acts on both the nucleic acid and the capsid protein,

with a shift to protein denaturation at about 50°C, and specific inhibitors of this heat inactivation include anionic detergents, collagen and cystin, while volatile fatty acids and NH_3 increase inactivation (Popat *et al.*, 2010). Lowering the ionic strength stabilises DNA viruses such as parvovirus, while the opposite effect occurs for enteroviruses (Wigand *et al.*, 1981).

Table 3. *Thermal treatments for enteroviruses and parvoviruses in different biowaste materials*

Virus	Temperature/ Time	Material	Reduction $\log_{10}$	Reference
Bovine enterovirus	55°C/30 min	Cattle manure anaerob. dig[a]	≥7.7	(Monteith *et al.*, 1986)
Porcine parvovirus (PPV)	55°C	Biogas substrate anaerob. dig.	D[b] 13.5	(Lund *et al.*, 1996)
PPV	55°C	Fresh manure	D 12	(Elving, 2009)
PPV	55°C	Biogas substrate	D 4.8	(Holmqvist *et al.*)
Bovine parvovirus (BPV)	56°C	Sewage sludge anaerob. dig.	D 4.7	(Spillman *et al.*, 1987)
Swine vesicular disease virus (SVDV)	55-60°C pilot scale	Raw slurry	~5-6	(Turner *et al.*, 1999b)
SVDV	65°C/2 min	Pig slurry	~6.5	(Turner & Williams, 1999)
SVDV	56°C/60 min	Pig slurry	2.3	(Herniman *et al.*, 1973)
	60°C/10 min		≥6.5	
Bovine enterovirus	70°C/30 min	Cattle manure, digested	≥6.3	(Monteith *et al.*, 1986)
PPV	70°C/60 min	Manure	0.6	(Lund *et al.*, 1996)
PPV	70°C/60 min	Manure/ household waste	1.4	(Lund *et al.*, 1996)
BPV	70°C/60 min	Slurry	2.6	(Böhm, 2005)
PPV	70°C	Biogas substrate	D 0.75	(Holmqvist *et al.*)
BPV	70°C/60 min	Liquid manure	0.8	(Srivastava & Lund, 1980)
BPV	70°C/30 min	Sewage sludge	0.72	(Spillman *et al.*, 1987)
BPV	80°C/15 min	Liquid manure	3.8	(Srivastava & Lund, 1980)

[a]during anaerobic digestion; [b]D-value, i.e. time (h) to reduce the virus titre by 1 $\log_{10}$

Thus, the character of the biowaste influences the rate of virus inactivation. Ammonia formation during anaerobic digestion could be the reason for the greater reduction in bovine enterovirus at 55°C for 30 min

(Monteith *et al.*, 1986) than during ordinary heat treatment of SVDV, another enterovirus, at 56°C for 60 min (Herniman *et al.*, 1973) (Table 3).

After heat treatment at 70°C for 60 min, inactivation of the parvoviruses ranges from 0.6 to 2.6 $\log_{10}$, and complete inactivation has not been reported even at 80°C for 15 min (Table 3), showing that the parvoviruses are very thermostable.

Chemical treatments

Chemicals act by different inactivation mechanisms. Alkalis such as lime and sodium hydroxide act by protein denaturation, while aldehydes such as formalin act by alkylation of protein and nucleic acids (De Benedictis *et al.*, 2007). The action of chemicals depends on the organic content of the material and on the temperature (Poschetto *et al.*, 2007).

Ammonia treatment

Ammonia (NH_3) treatment is an example of a chemical virus inactivation process. Ammonia in the form of ammonium salts is a common fertiliser. However, NH_3 in the uncharged form is toxic to living organisms such as humans, animals, plants and microorganisms (Warren, 1962). Cell membranes and other biological membranes such as virus coatings are freely permeable to the uncharged form, the NH_3 molecule.

Ammonia exists in ionised and non-ionised forms in water. Equation 1 shows the equilibrium between these in a simplified form. The equilibrium is dependent on the temperature and the pH, as the dissociation constant (pK_a value), the pH value at which the NH_3 molecule and its ionised form are present in equal amounts, varies with the temperature in the medium (Equation 2). If total ammonia and pH are measured, the pK_a value at each temperature can be used to calculate the fraction of NH_3 present (Equation 3) (Emerson *et al.*, 1975).

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \qquad \text{(Equation 1)}$$

$$pK_a = 0.09018 + 2729.92/T \qquad \text{(Equation 2)}$$

$$f = 1/(10^{pKa-pH} + 1) \qquad \text{(Equation 3)}$$

From Equation 2 it can be seen that the pK_a value decreases with increasing temperature T, and from Equation 3 that the fraction of NH_3 increases with increasing pH. If pK_a and pH are the same (equilibrium), the fraction of NH_3 is 50%.

Ammonia has been shown to inactivate poliovirus, an enterovirus possessing ssRNA, by cleavage of the viral RNA genome in intact virus particles. Suggested mechanisms of action of NH_3 are stimulation of the nuclease activity of viral capsid proteins/natural RNases, or pH increase inside the virus particle promoting alkaline hydrolysis, resulting in degradation of the viral RNA (Ward, 1978). Moreover, the inactivation is irreversible (Ward & Ashley, 1977a).

The virus-inactivating effect of NH_3 is evident in inactivation of bovine enterovirus in aerated pig slurry by addition of 43 mM NH_3 at 47°C, a temperature where no heat inactivation of this virus occurs (Wekerle & Albrecht, 1983). This effect has also been reported regarding SVDV inactivation in alkaline pig slurry (Turner *et al.*, 1999a). Ammonia inactivation has been investigated in human urine (Vinneras *et al.*, 2008), and inactivation by urea in faeces (Nordin *et al.*, 2009b; Vinnerås *et al.*, 2003). However, NH_3 leads to faster inactivation in manure compared with urea when equal amounts are added, as shown regarding bacteria (Ottoson *et al.*, 2008a). Ammonia treatment has been shown to be effective regarding eggs of the parasite *Ascaris* (Nordin *et al.*, 2009a). Urea treatment to eliminate bacteria has been evaluated in pig manure (Vinneras, 2007), as well as in cattle manure (Park & Diez-Gonzalez, 2003). Addition of NH_3 as a sanitising agent also increases the fertiliser value of the treated waste, through available nitrogen.

Other chemical methods

Chemicals used in biowaste management to reduce the virus content include calcium hydroxide (lime), sodium hydroxide and formalin (Turner & Burton, 1997). Liming is often used to treat slurry, but the treated slurry is less suitable as a fertiliser, as it may contain up to 2% sodium or calcium hydroxide (Turner *et al.*, 2000). However, it can be used in neutralising acidic soils (Koch & Euler, 1984). The use of 5% w/w quicklime on hatchery waste has been tested using the picornavirus ECBO as a standard, with a recommended treatment time of 3-7 days at ambient temperature and pH 12 (Philipp *et al.*, 2007).

In the event of an epizootic outbreak, e.g. of avian influenza or Newcastle disease, harsher methods are recommended, such as sodium hydroxide or formalin (Anon, 2009). New chemicals and methods are being developed, e.g. methylisothiocyanate, which has been shown to be effective against low pathogenic avian influenza H5N2 and even against the very stable virus Birnavirus (Gay & Mundt). In Denmark, a method involving

acidification of chicken pulp with sulphuric and lactic acid has been approved by the veterinary authorities and has been found to completely inactivate low pathogenic avian influenza H5N2 (Kabell *et al.*, 2009).

Model viruses

In the international guidelines on viral safety for biotechnology products (Anon, 1998), three different categories of model viruses are defined. For virus inactivation in biowaste, these definitions could be used.

Relevant model virus

If an identified virus is present in the material, the actual virus or a virus from the same subgroup should be used. In accordance, the relevant viruses used in our studies were swine vesicular disease virus and porcine parvovirus (Paper I), and highly pathogenic avian influenza virus (HPAIV) H7N1 and low pathogenic avian influenza (LPAIV) H5N3 (Paper II).

Specific model virus

If a relevant virus is not available, e.g. not cultivable, a specific model virus could be used. This virus should be from the same virus family or genus as the identified virus, and thus have similar physico-chemical properties. The specific model viruses used in Paper II were bovine parainfluenza virus (BPIV) 3 (model for Newcastle disease virus (NDV)) and feline coronavirus (FCoV) (model for avian infectious bronchitis virus (IBV)). According to the definitions, feline calicivirus (FCV) (Paper II) would be considered a non-specific model virus, but it could be considered a specific model virus for avian hepatitis E virus (AHEV), which was formerly in the same virus family, the Caliciviridae (Anon, 2005a).

Non-specific model virus

In order to determine the robustness of the virus inactivation process, non-specific model viruses having differing physico-chemical properties, preferably viruses having high resistance to physical or chemical treatments, may be used, as also stated in (EU, 2006). The PPV used in the heat treatment studies (Paper I) could be considered a non-specific model for stable naked viruses.

Bacteriophages

Bacteriophages are viruses that infect bacteria, and have a similar structure to animal viruses. They exhibit a range of physico-chemical properties, with some representatives being enveloped (Adcock *et al.*, 2009).
Bacteriophages are good virus models because they are harmless to humans, can be grown to high titres and are cheap and easy to analyse. In biowaste materials, several investigations have been performed using bacteriophages as indicators. For example, (Ottoson *et al.*, 2008b) used *Enterobacteria* phages MS2 and ϕX174, and *Salmonella* Typhimurium phage 28b to evaluate the sanitisation effects of NH_3 in mesophilic anaerobic digestion at an NH_3 concentration of 46 mM. However, the bacteriophages proved to be too conservative for this approach. In urine, the same bacteriophages have been used to assess inactivation at different temperatures (Vinneras *et al.*, 2008), with the assumption that inactivation rates are very slow below 20°C. In faeces, urea treatment resulting in 8000 ppm NH_3 gave a D-value (time to reduce the viral population by 1 $\log_{10}$, 90%) of 7.5 days for bacteriophage 28b at 20°C (Vinnerås *et al.*, 2003), while a similar D-value was found in faeces with 1% w/w urea addition, resulting in 200 mM NH_3, at 34°C (Nordin *et al.*, 2009b).

(Holmqvist *et al.*) used 28b as an indicator for heat inactivation in biogas substrate and found a D-value of 28 days at 56°C. Bacteriophages have also been evaluated regarding biological treatment (Ottoson *et al.*, 2006) and as monitors of microbiological quality of sewage sludge (Mandilara *et al.*, 2006). In this context, MS2 and ϕX174 have been found to be useful indicators of enteric virus removal in inoculation trials in pilot sludge plants (Arraj *et al.*, 2005). In studies on virus survival in groundwater, MS2 has been used as a conservative indicator for enterovirus (Yates *et al.*, 1985).

Based on their similar properties, we used bacteriophage MS2 as a model for ssRNA viruses and bacteriophage ϕX174, which has circular ssDNA, as a model with different physico-chemical properties (Paper II). We used bacteriophage 28b as a representative of a heat-stable dsDNA bacteriophage (Paper I).

Virus persistence in the environment

If ABP containing viruses are applied to farm land, e.g. as a fertiliser, environmental transmission to susceptible animals, wildlife or man may occur. The risk depends on the degree of virus persistence in different

environmental matrices, such as manure, soil and water. In soil, the main factors are moisture and temperature.

For AIV, environmental factors that influence persistence include temperature, pH, organic matter and water salinity (De Benedictis *et al.*, 2007). In chicken manure, a LPAIV H7N2 was found to survive >20 days at 4°C, but was inactivated in <1 week at 15-20°C (Lu *et al.*, 2003). LPAIV generally have longer persistence than HPAIV (Scholtissek, 1985). However, laboratory trials have shown persistence of HPAIV H7N1 for more than one year in manure-amended sandy soil at 5°C, and at 22°C for 7 weeks (unpublished results). In contrast, lower persistence of about 3 days was shown for LPAIV H10N7 applied to the same soil in outdoor lysimeters, probably due to the inactivating effect of UV-light, which has been shown to be a potent inactivator (De Benedictis *et al.*, 2007).

Surface water or groundwater may play a role as a reservoir for AIV, and thus it is important to assess inactivation rates in water (Stallknecht *et al.*, 1990). Those authors showed a persistence rate for duck LPAIV in distilled water of up to 207 days at 17°C, and more than 1000 days at 4°C. However, using environmental water and HP H5N1, dose-dependent persistence was found to be up to 60 days at 20°C, with longer survival in brackish water than pond or river water, but shorter survival in unfiltered water, suggesting a biological inactivation factor (Domanska-Blicharz *et al.*).

Numerous factors influence the survival of enteroviruses in soil, e.g. humidity, virus adsorption, temperature, enzymatic degradation (aerobic microorganisms) and pH (Hurst *et al.*, 1980). Thus, the major factors for survival and migration are climate (rain, temperature), the nature of the soil (clay content and moisture-holding capacity) and virus type. Viruses have been reported to migrate vertically to 67 m depth, and horizontally as far as 408 m, and generally to be more persistent in groundwater than in surface water or seawater, with decay rates of up to 0.7 $\log_{10}$ day^{-1} at 23°C for poliovirus in groundwater (Yates *et al.*, 1985). Enterovirus survival rates of up to 180 days at 4°C have been reported for a sandy loam (Wekerle, 1986). (Ellis & McCalla, 1978) reported persistence of enteroviruses for 25-170 days in soil, depending on soil type, pH, temperature and humidity. Enzymatic degradation of viral RNA by RNase activity has been shown in picornaviruses at temperatures of about 35°C (Gauntt, 1974), while in the case of poliovirus, specific bacterial enzymes have antiviral properties (Deng & Cliver, 1992). After spreading of sludge on arable land, enterovirus was found to be inactivated within two weeks, with a reduction of about 2.4 $\log_{10}$, according to PCR analysis (Pourcher *et al.*, 2007). The shorter

duration of persistence in that case was probably due to microorganism activity.

Swine hepatitis E virus can survive in manure for more than 2 weeks, both at 4°C and room temperature (USEPA, 2003). (Johansson *et al.*, 2005) showed that porcine parvovirus could persist in soil with minimal reduction for up to 50 days.

Materials and methods

For full details of the materials and methods used, see the relevant sections in Papers I and II.

Experimental setup

For the thermal treatment (Paper I), 200 mL volumes of substrate were inoculated with the relevant microorganism at a ratio of 1:10 to a final concentration of 7-8 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ g^{-1}, and kept in a monitored water bath at 55°C or 70°C. Sampling times were 0, 30 min and 60 min, including a heating-up time of 14-20 min, to mimic a batch experiment at a biogas plant.

For the ammonia study (Paper II), 0.9 g portions of hatchery waste were spiked with 0.1 mL of the relevant microorganism to a final concentration of 5-7 $\log_{10}$ $TCID_{50}$ g^{-1}, and thereafter with NH_3 to a concentration of 0.25, 0.5 and 0.75% w/w. The tubes were kept at 5, 14 or 25°C for 0-72 hours, and sampled 3-6 times depending on inactivation rate, and parameters studied were pH and total NH_3 nitrogen.

As controls in both Paper I and II, virus-spiked substrate (hold control) and cell culture medium (CCM control) were kept at room temperature (Paper I) or at the experimental temperature (Paper II). The reason for the CCM control was to determine the titre of the virus batch used and the effect of the incubation time and temperature, and by comparisons with the zero samples in the relevant materials, to calculate the virus recovery. The reason for the hold control was to determine the adsorbing/inactivating effect of the materials at the relevant temperatures and experimental periods.

Microorganisms

The viruses and bacteriophages were chosen based on their relevance as pathogens, or as models for other defined viral pathogens, depending on their physico-chemical properties. Table 4 shows an overview of the microorganisms used in the studies, and their properties. The microorganisms were propagated using their respective cell line/bacteria to titres of 7-9 $\log_{10}$ $TCID_{50}$ mL^{-1} for the viruses, and a titre of approximately 10 $\log_{10}$ plaque- forming units (PFU) mL^{-1} for the bacteriophages.

Biowaste

The substrate used in Paper I was untreated biowaste collected at a biogas plant, and consisted of Category 3 ABP materials, e.g. manure, blood, fat etc., together with waste from food industries and biological waste from households, separated at source. The hatchery wastes (HW) used in Paper II consisted of eggshells and tissue from developing embryos, and had a dry matter content of about 60%.

Microorganism analyses

All reactions were stopped on ice. The substrate samples (Paper I) were centrifuged at 7000 g for 2 min (Turner *et al.*, 1999c), gel-filtered through Sephadex G-25 in CCM to remove cytotoxic low molecular contaminants, and sterile-filtered using 0.45 μm filters. The HW samples were diluted 10-fold in the respective CCM and mixed for 1 min on vortex, followed by centrifugation at 3000 g for 10 min at 4°C and gel filtration as above. As all CCM for extractions, gel filtrations and titrations included antibiotics and antifungicides, these suppressed the action of bacteria and fungi on the cell cultures. Titrations were performed in serial dilutions in CCM using the respective cell line, analysing 8 replicates per dilution. The plates were incubated at 37°C in 5% CO_2 before evaluation of cytopathogenic effect (CPE), proceded by an immunoperoxidase assay for PPV (Paper I). Virus titres were calculated according to (Kärber, 1931), and expressed as $\log_{10}$ $TCID_{50}$ g^{-1}. For the bacteriophages, the double-agar method (Adams, 1959) was used and the titres were expressed as $\log_{10}$ PFU g^{-1}.

Table 4. *Viruses and bacteriophages used in Papers I and II, and properties of their virus families (Anon, 2005b), and cell lines/bacteria used for analyses*

Virus/ bacteriophage	Shape	Virus family	Size (nm)	Envelope	Cell line/bacteria	Genome	
						Type	Size (kb)
Porcine parvovirus[a] (I)	Icosahedral	Parvoviridae	18-26	No	Pig Kidney (PK)15[l]	ssDNA	4-6
Swine vesicular disease virus[b] (I)	Icosahedral	Picornaviridae	~30	No	Instituto Biologico-Renal- Swine (IB-R-S)-2[m]	+ssRNA	7-8.8
Avian influenza virus[c] (II)	Spherical/ pleomorphic	Orthomyxo-viridae	80-120	Yes	Madin Darby Canine Kidney (MDCK)[n]	segmented -ssRNA	10.0-14.6
Bovine parainfluenza virus 3[d] (II)	Spherical/ pleomorphic	Paramyxoviridae	~150	Yes	Bovine Turbinate (BT)[o]	-ssRNA	15-18.4
Feline coronavirus[e] (II)	Spherical	Coronaviridae	120-160	Yes	Felis Catus Whole Foetus (FCWF)[p]	+ssRNA	27.6-31
Feline calicivirus[f] (II)	Icosahedral	Caliciviridae	27-40	No	FCWF	+ssRNA	7.4-8.3
Enterobacteria phage MS2[g] (II)	Spherical/ icosahedral	Leviviridae	26	No	*Salmonella enterica* strain WG49 (ATCC 700730)	+ssRNA	3.5-4.3
Enterobacteria phage ϕX174[hi]	Icosahedral	Microviridae	~30	No	*Escherichia coli* strain C (ATCC 13706™)	circular +ssDNA	4-6
Salmonella Typhimurium phage 28B[k] (I)[j]	Isometric icosahedral with tail	Podoviridae	60-65	No	*Salmonella* Typhimurium phage type 5[q]	dsDNA	38-42

kb=kilobase; -ss=negative sense single stranded; +ss=positive sense single stranded; [a]strain 893/76; [b]strain 27/72; [c] HPAIV A/turkey/Italy/1387/00(H7N1) and LPAIV A/mallard/Sweden/1174/05(H5N3); [d]strain 1878/88, SVA; [e]strain DF2 (American type culture collection (ATCC) VR-2004); [f]strain 2280 (ATCC VR-2057); [g]ATCC 15597-B1; [h]ATCC 13706-B1; [i]only in thesis; [j](Lilleengen, 1948); [k]only in discussion; [l]ATCC CCL-33; [m]Pirbright laboratory, UK; [n]ATCC CCL-34; [o]SVA production; [p]ATCC CRL-2787; [q]Swedish Institute for Infectious Disease Control

Cytotoxicity and virus interference assays

For the viruses, cytotoxicity and virus interference assays were performed in order to determine the effect of the biowaste materials on the sensitivity of the detection systems (cell lines). The materials were treated as described above for microorganism analysis, and then the resulting effluent and its dilutions were added to the relevant cell line, incubated for the stipulated time, followed by microscopic evaluation of cytotoxicity. The relevant virus was titrated in different dilutions of effluent starting with the lowest non-cytotoxic dilution and analysed as above. The virus titres were compared with virus titrated in CCM only. Thus, the limit of detection where no virus was found could be calculated according to the formula: $c = -\ln p/V$, where 1-p is the 95% probability that the sample is free of infectious virus ($p<0.05$), v is the tested volume, and c is the virus concentration (Blumel *et al.*, 2002).

Evaluation methods

The evaluation methods were based on the infectivity of the virus using their relevant detection system, in this case a susceptible cell line showing cpe. When the virus was non-cytopathogenic, the infection was combined with an immunological method, such as immunoperoxidase staining (Paper I). The titre values obtained reflect the sensitivity of the detection system to the virus. All living virus particles are not necessarily detected, and thus virus reduction factors were calculated to estimate the efficiency of the inactivation process.

Virus reduction factors (Paper I)

Log_{10} reductions were calculated from the individual titre values, with the initial value taken at the start. Virus reduction factors R were thus calculated according to (Anon, 1998) using the formula: $10^R = v' \times 10^{a'} / v'' \times 10^{a''}$, where v is the starting/final volume, and a is the starting/final titre.

Regression analysis (Paper II)

For each microorganism, NH_3 concentration and temperature, the individual log_{10} titre values obtained were plotted in a diagram as a function of time, and linear regression analysis was used to determine the inactivation rate constant in the different treatments. D-values (the time required to reduce the population by 1 log_{10}, 90%) were derived by taking the reciprocal of k and a 95% confidence interval of D was calculated from the

t-distribution of the standard error from the regression analysis. The time needed for the specific reductions of 3 and 12 $\log_{10}$ were extrapolated using the upper 95% confidence interval.

Large-scale study using hatchery waste

The study examining NH_3 inactivation of hatchery waste (HW) included a large-scale trial to test the recommendations drawn up in a hatchery during normal production, where liming was usually carried out.

The mixing tanks were thoroughly rinsed with tap water before the trial to remove every trace of lime, and the pH was checked to ensure neutrality. In a vacuum container of about 3 m^3 volume, about 0.5 m^3 of HW were continuously spiked with 5 L MS2 bacteriophage, diluted to 50 L, to mimic the laboratory trials. Duplicate initial samples were taken from the top and bottom of the tank. A 60 L volume of 28% NH_3 solution was diluted to 200 L using tap water, and gradually introduced to the HW. After 3 hours, one-tenth of the NH_3 mixture had been introduced into the HW, and a NH_3 concentration of about 0.2 % w/w had been achieved. From then on, ammonia was introduced at a faster rate, together with HW up to 2 m^3 volume, reaching a final ammonia concentration of about 0.6 % w/w after 6 h. During the experiment, duplicate samples were taken at 0, 2, 4, 6, 7 and 24 h, from the bottom of the tank as well as the top, to verify thorough NH_3 mixing. Hold control samples were kept in HW with deionised water instead of NH_3. Samples for analysis of NH_3 nitrogen and pH were stored cool before analysis, and samples for bacteriophage analyses were stored and transported frozen. Parameters analysed in duplicate for each sampling occasion were: total NH_3 nitrogen, pH, temperature and MS2 titre.

Main results

Thermal inactivation (Paper I)

The results from the three experiments on PPV in biogas substrate after the different thermal treatments are shown in Table 5. The mean reduction in virus titre (n=6) for the hold control sample was 0.5 $\log_{10}$, reflecting the mean adsorption/inactivation capacity of the substrate after the duration of the experiment, as the control in CCM was stable. The mean virus recovery from the substrate was 47% compared with the positive control in CCM (n=6).

Table 5. *PPV titre ($\log_{10} TCID_{50}\, g^{-1}$) in thermal experiments at 55°C and 70°C using three different batches of biogas substrate*

Time	PPV 1		PPV 2		PPV 3	
(min)	55°C	70°C	55°C	70°C	55°C	70°C
CCM (0)	7.1	7.1	7.1	6.8	7.7	7.3
BS(0)	6.4	6.2	6.4	6.9	7.6	7.6
BS (30)	4.9	3.1	5.6	4.5	6.9	5.4
BS (60)	4.3	2.9	5.4	3.9	6.4	4.3
HOLD (60)	5.7	5.6	6.1	6.1	7.6	7.1
CCM (60)	7.4	6.9	6.7	6.9	7.6	7.4
Reduction[a] (60)	1.4	2.7	0.7	2.2	1.2	2.8

HOLD = hold control, virus in biogas substrate (BS) at r.t.; CCM = virus in cell culture medium at r.t. ; [a]Virus reduction after 60 min due to heat treatment

At 55°C, the mean reduction was 1.0 and 1.4 $\log_{10}$ at 30 min and 60 min, respectively. At 70°C, the corresponding values were 2.6 and 3.2 $\log_{10}$. As a mean adsorption of 0.5 $\log_{10}$ was shown, the actual mean reductions due to

heat treatment after 60 min, were 1.1 and 2.6 $\log_{10}$, for 55°C and 70°C, respectively.

Table 6. *SVDV titres ($\log_{10}TCID_{50}$ g^{-1}) in thermal experiments at 55°C and 70°C using three different batches of biogas substrate*

Time	SVDV 1		SVDV 2		SVDV 3	
(min)	55°C	70°C	55°C	70°C	55°C	70°C
CCM (0)	8.1	8.2	8.4	8.4	8.7	8.4
BS(0)	8.1	7.3	8.4	7.9	8.4	8.8
BS (30)	6.5	≤[a] 1.7	6.9	≤ 1.7	7.3	≤ 2.4
BS (60)	6.0	≤ 1.7	6.3	≤ 1.8	6.9	≤ 2.4
HOLD (60)	7.8	7.8	7.9	7.7	8.3	8.4
CCM (60)	8.2	8.2	8.1	8.2	8.6	8.1

HOLD = hold control, virus in biogas substrate (BS) at r.t.; CCM = virus in cell culture medium at r.t.; [a]detection limit varied according to amount of sample tested

The results from the three experiments for SVDV in biogas substrate after the different thermal treatments are shown in Table 6. The mean reduction in virus titre (n=6) for both the hold control and the CCM control was 0.2 $\log_{10}$, showing no adsorption/inactivation capacity of the substrate throughout the experiment. The mean virus recovery from the substrate was 60% compared with the positive control in CCM (n=6). At 55°C, the mean reduction was 1.4 and 1.9 $\log_{10}$ at 30 and 60 min, respectively. At 70°C, the mean reduction was ≥6.1 $\log_{10}$ after 30 min.

Ammonia inactivation (Paper II)

Tables 7 and 8 show NH_3 additions and recorded pH and NH_3 concentrations at the respective temperatures tested, for naked and enveloped viruses, respectively. The inactivation rate (95% confidence interval), and the corresponding D-value are also shown. All viruses were efficiently inactivated already at 0.25% w/w NH_3 addition, at rates of ≥0.12$\log_{10}$ h^{-1}. Studies on the enveloped viruses (Table 8) showed that at 5°C BPIV 3 demonstrated the highest stability, while at 14°C and 25°C, HPAIV H7N1 was the most stable. This is also shown in Figure 1, where H7N1 is compared with the naked virus FCV and bacteriophage MS2. The inactivation rate for MS2 was lower that that of all other viruses tested, so MS2 is a good indicator of virus survival.

Table 7. *NH_3 additions (% w/w) and recorded NH_3 concentrations (mmol kg^{-1}) at the temperature and pH values used in the study. Inactivation rate for the naked viruses is also given (log_{10} h^{-1} ± standard error) and the corresponding D-value (time for 1 log_{10} reduction, hours). R^2 value indicates curve fit*

Virus/ phage	Temp (°C)	pH	NH_3 %w/w	NH_3 mmol kg^{-1}	Inactivation rate ±SE (log_{10} h^{-1})	n	D (h)	R^2
FCV	5	8.0	0	2.3	0.050±0.020	10	20	0.44
		9.2	0.25	43	0.18±0.019	10	5.6	0.91
		9.5	0.5	119	0.29±0.024	10	3.4	0.95
	14	8.0	0	4.5	0.070±0.034	10	14	0.35
		9.2	0.25	74	0.41±0.032	10	2.4	0.96
		9.5	0.5	186	0.5±0.04	10	2	0.95
	25	8.0	0	9.8	0.094±0.01	10	11	0.91
		9.2	0.25	121	0.37±0.065	10	2.7	0.80
		9.5	0.5	268	0.77±0.084	8	1.3	0.93
MS2	5	8.0	0	2.3	0.0064±0.0018	8	156	0.69
		9.2	0.25	43	0.063±0.0040	10	16	0.97
		9.5	0.5	119	0.13±0.0052	8	7.7	0.99
		9.7	0.75	190	0.17±0.0024	8	5.9	1.0
	14	8.0	0	4.5	0.0080±0.0012	14	125	0.78
		9.2	0.25	74	0.069±0.0036	14	14	0.97
		9.5	0.5	186	0.13±0.014	12	7.7	0.90
		9.7	0.75	274	0.19±0.021	12	5.3	0.89
	25	8.0	0	9.8	0.018±0.0021	7	56	0.94
		9.2	0.25	121	0.15±0.02	10	6.7	0.87
		9.5	0.5	268	0.43±0.043	7	2.3	0.95
		9.7	0.75	363	0.79±0.045	4	1.3	0.99

All inactivation rates increased significantly with NH_3 addition and temperature. The final virus reductions were ≥4.2 (LPAIV), ≥5.7 (HPAIV), ≥4.6 (BPIV 3), ≥4.7 (FCV) and ≥5.0 (FCoV) log_{10} $TCID_{50}$ g^{-1} and ≥5.0 PFU g^{-1} for MS2, depending on the initial titres. Bacteriophage recovery from the HW for MS2 and ϕX174 was 100%. The virus recoveries were: H5N3: 79%, H7N1: 32%, FCV: 100%, FCoV: 100%, and BPIV 3 :38%.

Bacteriophage ϕX174 did not show any significant inactivation from ammonia treatment, at most 0.4 log_{10} after 72 h at 25°C.

Table 8. *NH_3 additions (% w/w) and recorded NH_3 concentrations (mmol kg^{-1}) at the temperature and pH values used in the study. Inactivation rate for the enveloped viruses is also given (log_{10} h^{-1} ± standard error) and the corresponding D-value (time for 1 log_{10} reduction, hours. R^2 value indicates curve fit*

Virus/ phage	Temp (°C)	pH	NH_3 %w/w	NH_3 mmol kg^{-1}	Inactivation rate ±SE (log h^{-1})	n	D_t (h)	R^2
LPAIV	5	8.0	0	2.3	0.088±0.018	8	11	0.80
		9.2	0.25	43	0.17±0.040	10	5.9	0.69
		9.5	0.5	119	0.48±0.055	8	2.1	0.93
		9.7	0.75	190	0.69±0.027	5	1.4	1.0
	14	8.0	0	4.5	0.11±0.022	8	9.1	0.81
		9.2	0.25	74	0.31±0.031	10	3.2	0.93
		9.5	0.5	186	0.47±0.062	8	2.1	0.91
		9.7	0.75	274	0.69±0.024	6	1.4	1.0
	25	8.0	0	9.8	0.35±0.023	8	2.9	0.97
		9.2	0.25	121	1.3±0.20	6	0.78	0.91
		9.5	0.5	268	1.3±0.23	6	0.79	0.88
		9.7	0.75	363	1.3±0.25	6	0.79	0.86
HPAIV	5	8.0	0	2.3	0.024±0.044	7	41	0.058
		9.2	0.25	43	0.15±0.030	13	6.7	0.68
		9.5	0.5	119	0.23±0.044	13	4.3	0.72
		9.7	0.75	190	0.31±0.049	13	3.2	0.79
	14	8.0	0	4.5	0.027±0.015	7	37	0.41
		9.2	0.25	74	0.25±0.027	13	4.1	0.88
		9.5	0.5	186	0.34±0.034	13	3.0	0.90
		9.7	0.75	274	0.43±0.042	13	2.3	0.91
	25	8.0	0	9.8	0.16± 0.048	7	6.3	0.69
		9.2	0.25	121	0.47±0.038	13	2.1	0.92
		9.5	0.5	268	0.71±0.049	13	1.4	0.95
		9.7	0.75	363	0.97± 0.092	11	1.0	0.92
BPIV-3	5	8.0	0	2.3	0.053±0.015	12	19	0.54
		9.2	0.25	43	0.12±0.033	12	8.3	0.58
		9.5	0.5	119	0.16±0.042	12	6.3	0.59
		9.7	0.75	190	0.17±0.041	12	5.9	0.64
	14	8.0	0	4.5	0.15±0.029	8	6.7	0.81
		9.2	0.25	74	0.29±0.04	8	3.4	0.91
		9.5	0.5	186	0.77±0.029	6	1.3	0.99
		9.7	0.75	274	>1.5	4		1
	25	8.0	0	9.8	0.17±0.02	10	5.9	0.88
		9.2	0.25	121	0.41±0.080	10	2.4	0.76
		9.5	0.5	268	1.5±0.14	6	0.67	0.97
		9.7	0.75	363	>1.5	4		1
FCoV	5	8.0	0	2.3	0.077±0.017	10	13	0.71
		9.2	0.25	43	0.33±0.065	10	3.0	0.77
		9.5	0.5	119	0.48±0.082	10	2.1	0.81
	14	8.0	0	4.5	0.028±0.016	10	36	0.28
		9.2	0.25	74	0.39±0.077	10	2.6	0.76
		9.5	0.5	186	0.84±0.084	7	1.2	0.95
	25	8.0	0	9.8	0.30±0.064	10	3.3	0.73
		9.2	0.25	121	1.6±0.17	6	0.63	0.96
		9.5	0.5	268	1.6±0.18	6	0.63	0.95

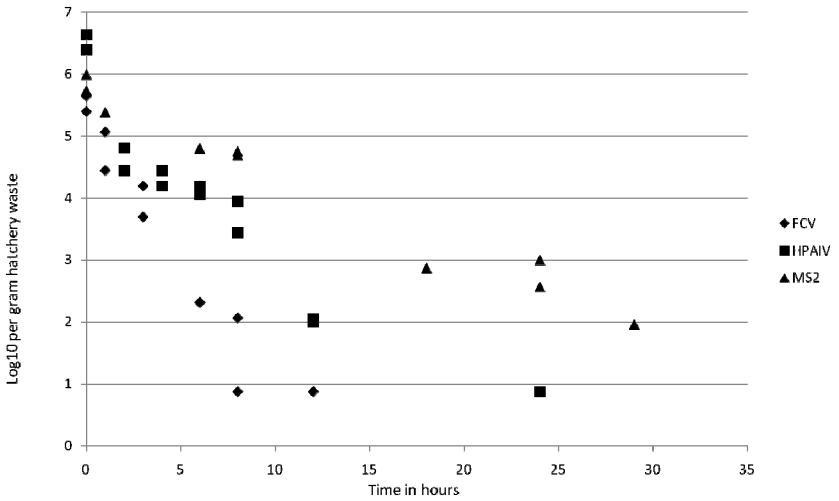

Figure 1. Inactivation time (hours) at 14°C and 0.5% w/w NH_3 (186 mM) for feline calicivirus (FCV), highly pathogenic avian influenza (HPAIV) H7N1 and *Enterobacteria* phage MS2 (MS2). Titres in Tissue culture infectious dose $(TCID)_{50}$ g^{-1}. Detection limit is 0.9 $\log_{10}$ g^{-1} .

Large-scale study at a hatchery

Table 9 shows the volume and the mean titres of MS2 on each sampling occasion in the large-scale ammonia treatment trial at the hatchery, which used HW soaked with MS2 bacteriophage.

Table 9. *Total MS2 titre (PFU log_{10}), recorded NH_3 concentration (mmol kg^{-1}), pH and temperature (°C) in the added HW (volume added shown in m^3) at 0, 2, 4, 6, 7 and 24 hours in the large-scale trial using MS2 at the hatchery*

Time (h)	HW (m^3)	Total MS2 (PFU log_{10})	NH_3 mmol kg^{-1}	pH	Temp°C
0-T[a]	0.5	13.1	0.22	7.4	20
0-B[b]	0.5	13.2	0.19	7.3	20
2-T	0.5	12.0	32	9.2	21
2-B	0.5	11.8	36	9.2	21
4-T	1	7.7	99	9.8	22
4-B	1	<	363	10.3	22
6-T	2	<	214	9.9	20
6-B	2	<	353	10.3	20
7-T	2	<	160	10	21
7-B	2	<	146	10	22
24-T	2	<	81	10	19
24-B	2	<	375	9.9	19

Mean of two samples; [a]= top of the tank; [b]=bottom of the tank; < = below detection limit 5.9 log_{10}; PFU =plaque forming units

Figure 2 shows the inactivation rate of MS2 in HW, together with the ammonia concentration during the large-scale trial. As the volume of HW varied through new additions (Table 9), the MS2 concentration is expressed in total log_{10}.

The detection limit was 5.9 log_{10} $tank^{-1}$, due to the large volume of HW. At time 6 h, NH_3 had been added to about 0.6 %w/w, resulting in 214 mmol kg^{-1} for the top sample. The inactivation rate of MS2 was 1.3 log_{10} per hour at a temperature of 19-22°C. The control with HW and water did not show any significant reduction up to 24 hours.

Cytotoxicity and viral interference assays

For PPV, the 3^{-5} dilution was shown not to be interfering and this was sufficient, as PPV did not show a reduction down to the detection limit, even at 70°C for 60 min. The undiluted substrate could be used for virus analysis regarding H7N1 and H5N3. Regarding the other viruses the substrate had to be diluted 1:3 or 1:5 to get a valid assay. By analysing larger volumes, the detection limit ranged between 0.9 and 2.4 log_{10} $TCID_{50}$ g^{-1}. The reason for the different susceptibility to the substrates was differing substrate composition and differing sensitivity and growth properties of the cell lines.

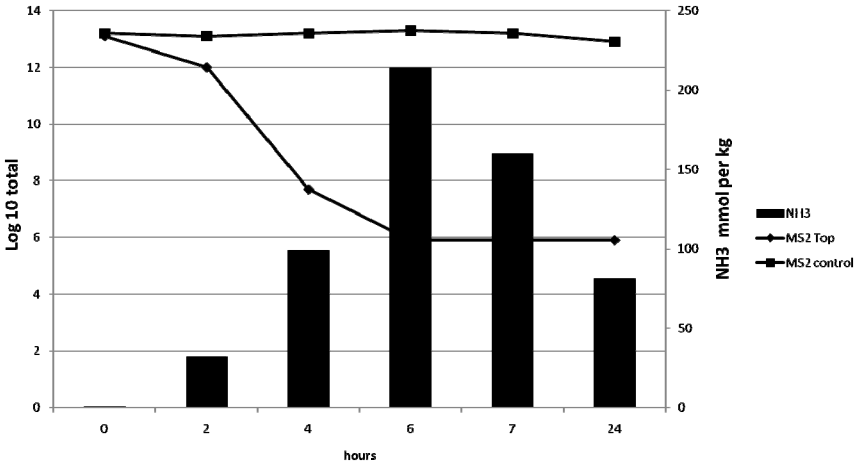

Figure 2. Actual ammonia concentration (mmol kg^{-1}) in the large-scale trial for the sample taken at the top of the tank (worst case conditions). Titres of bacteriophage MS2 are in PFU.

Discussion

Biowaste, e.g. ABP, contains valuable nutrients and thus the optimal use for these would be as a fertiliser, recycling the nutrients into the food chain. Ammonia inactivation (Paper II) was shown to be highly effective regarding the viruses tested. However, the results obtained for thermal inactivation (Paper I) raise some concerns regarding inactivation of the most heat-stable viruses.

Thermal inactivation (Paper I)

The thermal inactivation experiments (Paper I) showed that SVDV, which is known to be excreted in pig manure and is used as an indicator for enterovirus inactivation, is sufficiently reduced after heat treatment at 70°C for 30 min. As enteroviruses have been shown to be prevalent in animal waste, such as manure, this result is encouraging.

The experiments were performed in order to assess the inactivation effect of thermal treatment at 70°C for 60 min, the proposed requirement for Category 3 ABP materials at that time (EU, 2002). The recommended reduction was 4 $\log_{10}$ based on the expected amount of enteroviruses (Carrington, 2001). We tested the thermally stable virus PPV and the important epizootic virus SVDV, a differential diagnosis for foot-and-mouth disease virus. The thermal treatments were performed in biogas substrate at 70°C for 30 and 60 min, simulating a batch process in a biogas plant.

Experiments were also performed at 55°C, as this temperature is often used in thermophilic processes, and our results showed a very good correlation to other thermal studies using SVDV, which has previously been investigated regarding inactivation in spiked pig slurry (Table 3)(Figure 3). In our experiments at 55°C, we found mean reductions of 1.4 and 1.9 $\log_{10}$, after 30 and 60 min, respectively. This is in good agreement with previous

studies at 56°C (Herniman *et al.*, 1973), which found inactivations of 1.8, 2.3 and 3 $\log_{10}$ after 30, 60 and 120 min, respectively. At higher temperatures, the same authors found complete reduction of ≥6.5 $\log_{10}$ at 60°C after 10 min (Figure 3), and noted that SVDV was more stable in slurry than in milk, probably due to matrix differences. At 70°C, our studies showed a mean reduction of ≥6.1 $\log_{10}$ after 30 min, comparable to the reduction of about 6.5 $\log_{10}$ reported at 65°C for 2 min (Turner & Williams, 1999), showing the consistency of thermal treatment regarding SVDV.

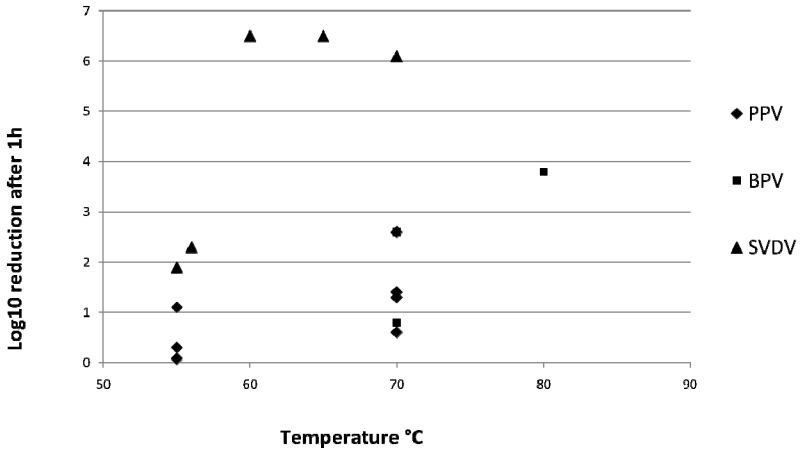

Figure 3. Log_{10} reduction for Porcine parvovirus (PPV), Bovine parvovirus (BPV) and Swine vesicular disease virus (SVDV), after 1h in different biowaste materials. SVDV reductions at 60°C, 65°C and 70°C are after 10, 2 min and 30 min, respectively.

For PPV at 55°C (Figure 3), we obtained a mean reduction of 1.1 $\log_{10}$ after 60 min, due to heat. (Lund *et al.*, 1996) found longer survival times for PPV at 55°C, tested in laboratory biogas reactors fed with manure/manure with 20% household waste (Table 3). They observed biphasic inactivation curves, with rates of 0.3 and 0.07 $\log_{10}$ h^{-1}. Similarly, (Elving, 2009) tested PPV in fresh cattle manure and obtained a rate of about 0.08 $\log_{10}$ h^{-1}, i.e. comparable to the lowest rate of Lund *et al.* (1996). Both these authors had similar biowaste, as Lund *et al.* (1996) used 75% cattle manure and 25% pig manure (no reported dry matter content), while the fresh cattle manure used by Elving *et al.* (2009) had a dry matter content of about 13%. For PPV at 70°C, we found a mean reduction of 2.6 $\log_{10}$ after

60 min, due to heat, with a calculated reduction of 1.3 $\log_{10}$ after 60 min. (Lund *et al.*, 1996) found a mean $\log_{10}$ reduction for PPV of 1.4 at 70°C for 60 min, using manure and household waste, and these authors opposed the requirement of a 4 $\log_{10}$ reduction as being too high for such a heat-resistant virus as PPV. Instead, they recommended the use of a picornavirus. Other studies using parvovirus in manure/slurry heat-treated at 70°C for 30-60 min showed reductions of 0.6-2.6 $\log_{10}$ (Table 3)(Figure 3).

In Paper I we did not characterise the biogas substrate, but it was in a liquid form and thus had a low dry matter content. Other differences in composition of the biowaste could influence results, since e.g. NH_3, H_2S and volatile fatty acids have inactivating effects (Lund *et al.*, 1996). Furthermore, as shown for enteroviruses, collagen and anionic detergents protect viruses (Popat *et al.*, 2010). We also had a heating-up time of about 14-20 min before time recording started, and this could have added some initial reduction. Our experiment was intended to mimic the conditions at the biogas plant, and evaluate the reductions in this context.

Nevertheless, our results regarding PPV indicate that other heat-stable viruses such as circoviruses would also survive these high temperatures. This is indeed the case, as porcine circovirus 2, which has been shown to be common in e.g. Sweden (Linné *et al.*, 2000) and can be present without clinical disease symptoms, has been shown to be very heat-stable. In a protein environment of 10% FBS, there was a 4.7 $\log_{10}$ reduction at 90°C for 15 min, while at 95°C for 5 s the virus was still viable, with a reduction of 5.6 $\log_{10}$ (Emmoth, 2005). Porcine circovirus is a naked virus of 17-21 nm, smaller than PPV, and with covalently closed, circular ssDNA, hence its stability (Anon, 2005b).

The naked dsDNA bacteriophage 28b tested in the thermal inactivation studies did not exhibit any significant inactivation after exposure to 70°C for 60 min and thus it is probably too heat-resistant to serve as an indicator regarding this treatment. This was also shown by (Holmqvist *et al.*), who found that 28b was six and seven times more heat resistant than PPV at 55°C and 60°C, respectively, indicating that the difference is increasing with temperature.

In conclusion, PPV showed a high stability during heat treatment, and did not reach the stipulated 4 $\log_{10}$ reduction, even at 70°C after 60 min, and bacteriophage 28b was even more heat resistant. In view of the revised ABP-regulation (EU 2006), which recommends parvovirus as an indicator organism for a 3 $\log_{10}$ reduction, this could lead to unnecessary high demands regarding biowaste from biogas- and composting plants, especially for treatment alternatives at lower temperatures. In contrast, SVDV, a

model for enterovirus, reached reductions of ≥5.6 - ≥6.4 $\log_{10}$ already after 30 min at 70°C, and thus is probably not a suitable model for other heat stable virus groups. This was also confirmed by (Lund *et al.*, 1996) for bovine enterovirus, with a >3.6 $\log_{10}$ reduction after 70°C for 60 min. The ideal model would be a microorganism, e.g. a bacteriophage, showing a heat resistance at this temperature between parvo- and enterovirus.

The results from the various thermal studies (Table 3) indicate a wide range of treatment times to reach a 3 $\log_{10}$ reduction. If PPV is used, most of the studies indicate that treatments at temperatures below 70°C will require very long treatment times, e.g. 55°C treatment would need 14 - 40 h fulfilling the 3 $\log_{10}$ target. If virus content is considered a risk in reusing ABPs, a further evaluation is required to specify the virus, depending on the kind of biowaste material used.

Ammonia inactivation of viruses in hatchery waste (Paper II)

At hatcheries today, the sanitisation treatment often used for HW is liming. As the lime is generally applied as a suspension in water, sediments may form and cause handling problems, while the high pH (>12) creates problems in the working environment. In order to provide an alternative treatment method, we tested ammonia inactivation in HW at different concentrations and temperatures. Acceptable virus reductions were shown for all ssRNA viruses tested, with inactivation rates significantly increasing with NH_3 addition and temperature. Interestingly, the enveloped viruses, which have not been tested previously in terms of NH_3 treatment, had similar reduction rates to the naked viruses. Figure 4, having logarithmated D-values for comparison, shows that orthomyxovirus (H7N1) have a D-value comparable to calicivirus, even at a high NH_3 concentration of 268 mmol kg^{-1}, while MS2 has always higher D-values. However, the other enveloped viruses have very low D-values at this NH_3 concentration.

Our results regarding the naked ssRNA viruses are in good agreement with earlier reports studying NH_3 inactivation. In our study at 25°C, pH 9.5 and 268 mmol kg^{-1} NH_3, MS2 had a D-value of 2.3 h, while FCV, which has a similar structure as Picornaviruses, has a D-value of 1.3 h. In similar conditions, (Ward & Ashley, 1977b) showed that at 21°C, pH 9.5 and an NH_3 concentration of 294 mM, six different Picornaviruses were inactivated >6 $\log_{10}$ in 24 h (D-value <4 h) (Figure 4).

Furthermore, (Cramer *et al.*, 1983) assessed the effect of NH_3 on bacteriophage f2, of the same family Leviviridae as MS2. In his experiments,

he observed a 4.5 times higher stability to NH_3 of f2, than for poliovirus, a Picornavirus. At a temperature of 20°C, pH 8.6 and an NH_3 concentration of 36 mM he found a D-value of 14 h for f2. In Paper II, MS2 demonstrated the same D-value at 14°C, pH 9.2 and 74 mmol kg^{-1} NH_3. In the same range, MS2 was 5.8 fold more stable than FCV, an ssRNA virus such as poliovirus (Figure 4). Moreover, in the same experiment, at pH 9 and an NH_3 concentration of 176 mM, (Cramer *et al.*, 1983) found a D-value of about 6 h for f2, where we, for MS2, demonstrated a D-value of 7.7 h, at 14°C, pH 9.5 and 186 mmol kg^{-1} NH_3. Bacteriophage f2 has also been used in experiments with nonaerated animal waste, where the effect of NH_3 was noted at 16°C and pH 8.7 (Pesaro *et al.*, 1995).

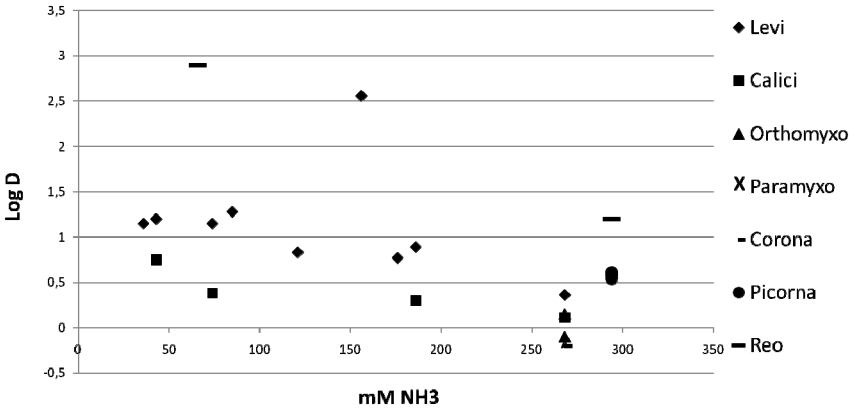

Figure 4. Log D-values (h) at different NH_3 concentrations (mM) or mmol kg^{-1} for HW, at temperature ranges of 5-25°C and pHs of 8.0-9.5, for viruses in different ssRNA virus families: Leviviridae (Paper II, (Chandran *et al.*, 2009; Vinneras *et al.*, 2008; Cramer *et al.*, 1983)), Calici-, Orthomyxo-, Paramyxo-, Coronaviridae (Paper II), Picornaviridae and Reoviridae (Höglund *et al.*, 2002; Ward & Ashley, 1977b).

They found that f2 had similar sensitivity to NH_3 as encephalomyocarditis virus, another ssRNA virus. These studies indicate that bacteriophages of this type are good models for naked ssRNA viruses regarding NH_3 inactivation. Moreover, in the two first studies mentioned the virus was tested in solution, whereas we used HW with an experimental dry matter content of 49%, indicating that NH_3 is effective in materials with varying dry matter content.

In comparison with studies concerning urine disinfection, where urea is the source of NH_3, we obtained diverging results. Our results at 74 mM NH_3, 14°C, and pH 9.2 and a D-value of 14 h for MS2, showed good correlation to (Chandran *et al.*, 2009), with a D-value for MS2 of <19 h at an NH_3 concentration of about 85 mM NH_3, at 15°C and pH 8.9 (Figure 4). However, in comparison with (Vinneras *et al.*, 2008), which at an NH_3 concentration of 156 mM NH_3, 24°C and pH 9.0, found a D-value for MS2 of 15±3 days, i.e. about 50-fold higher than our results, at an NH_3 concentration of 121 mmol kg^{-1}, 25°C and pH 9.2, with a D-value for MS2 of 6.7 h (Figure 4). However, (Vinneras *et al.*) found two inactivation rates for MS2 and ϕX174, a higher rate up to three days, which was excluded from the regression. We used NH_3 and this reaches a higher pH compared with urea, where the carbonate that formed may have buffered the material to give a lower pH at the same equimolar amount added (Ottoson *et al.*, 2008a), explaining some of the difference in inactivation rate. In contrast, the effect regarding bacteriophage ϕX174 in the same conditions was more consistent, as we found a D-value of about 25 days and (Vinneras *et al.*, 2008) a D-value of 12±10 days, i.e. in the same range. These results confirm that bacteriophage ϕX174 is very insensitive to NH_3 inactivation. It possesses circular ssDNA and this perhaps confers more resistance to inactivation, because NH_3 acts on the nucleic acid.

In conclusion, the MS2 bacteriophage proved to be an excellent model for inactivation of ssRNA viruses in the HW study. Its inactivation as regards ammonia addition and temperature correlated with that of all virus groups tested and it was inactivated at a lower rate, so it is suitable as a conservative indicator regarding NH_3 inactivation. In order to achieve a 3 $\log_{10}$ reduction for HW treatment, using MS2 as a representative virus model of a heat-stable virus under normal conditions, the recommendation is to add 0.25% w/w NH_3 plus storage for 2 days, or 0.5% w/w NH_3 plus storage for at least 31 h, at ≥14°C. However, a chemical process such as ammonia treatment also has to be validated using a parasite, such as *Ascaris suum*. Previous studies have shown high efficiency of NH_3 under similar conditions and concentrations for this parasite in human faeces (Nordin *et al.*, 2009a). This is important, as *A. suum* eggs have been shown to survive for 214 days in sewage sludge during storage at 21°C (Berggren *et al.*, 2005).

Other viruses, such as parvovirus or adenovirus, should also be tested, because double-stranded viruses such as reovirus or rotavirus seem to be less affected by NH_3. For example, in another study (Ward & Ashley, 1977b), reovirus was not as sensitive to NH_3 as ssRNA viruses. At 21°C, pH 9.5

and an NH_3 concentration of 294 mM, reovirus was inactivated by <2 log_{10} (D >12 h). Similarly, at 20°C, (Höglund *et al.*, 2002) saw no inactivating effect of NH_3 regarding rotavirus, of the Reoviridae virus family, in urine at pH 9 and 66 mM NH_3, with a mean D-value of 35 days, contributed to heat (Figure 4).

The large-scale trial in this thesis showed a higher inactivation rate regarding MS2 than the laboratory experiments. Despite the fact that the NH_3 concentration of the HW at the top of the tank declined after the mixing stopped, probably due to separation of lipid material, resulting in higher NH_3 concentration in the bottom of the tank, the final top sample at 24 hours had an ammonia concentration of 81 mmol kg^{-1} at 19°C and pH 10. As this is 74 mmol kg^{-1} recalculated to 14°C, the recommended treatment for 3 log_{10} reduction of a thermostable virus (EU, 2006), namely 0.25% w/w ammonia (74 mmol kg^{-1} in Table 7) and storage for 2 days at >14°C, could be used. The reason for the difference in inactivation rate is probably the higher temperature, as the inactivation is not totally linear between different temperatures, and also the fact that the mixing was very efficient under vacuum in the larger mechanised system.

The large-scale trial was performed through estimation of the volume of hatchery waste, which was about 2 m^3 final volume. Since 1 m^3 waste from this hatchery weighs about 1100-1200 kg, depending on humidity, this has to be taken into consideration for treatment recommendations. Nevertheless, faster inactivation of MS2 was observed in the large-scale trial. To prevent ammonia evaporation a closed container is necessary, and the amount of HW, degree of mixing, pH and temperature should be monitored. As regards problems in the working environment from NH_3 fumes, a proper mask/air protection is needed. This has to be evaluated in comparison to the problems with liming.

For notifiable diseases, this thesis showed that AIV of both low and high pathogenicity and virus model for NDV could be sufficiently inactivated by NH_3 treatment of HW. In an epizootic outbreak situation, it is important to have a practical and safe disinfection method for biosecurity reasons, as disinfection of e.g. ABP such as HW must be handled in a closed environment. In the event of an outbreak of HPAIV or any of the other viruses tested in our study, the recommended treatment of HW in order to achieve a 12 log_{10} reduction (http://www.pda.org) is addition of 0.5% w/w NH_3 and storage for 2 days at >14°C.

Biowaste management

Both studies presented in the papers investigated biowaste treatments in bench scale studies controlling the factors considered to be the most important for virus inactivation, heat and ammonia. By looking at the effect on viral reduction as a function of these factors, the idea is to give recommendations of temperature and/or ammonia concentration that should be kept in the process over a certain time to reach sufficient viral reductions.

In Paper II, we used regression analysis, and based on the virus inactivation rates at the different ammonia/temperature combinations, D-values were derived. Based on the D-values, treatment times to reach the desired $\log_{10}$-reductions were recommended, by using confidence intervals of 95% or 99%, to reach a sufficient safety margin for the purpose. Safety margins must be set due to e.g. inhomogeneity of the biowaste, and because that often the actual virus content is not known. In an epizootic outbreak situation, a larger safety margin might be desired than in normal biowaste management. In that case the upper 99% confidence interval of the D-value could be used to calculate the contact time after the specific ammonia addition and temperature.

However, to test the recommendations in large scale, it is inconvenient to use the actual animal virus, due to its pathogenicity. Instead, bacteriophages could be analysed in parallel in the laboratory trials, and if the bacteriophage is shown to have a lower inactivation rate than the pathogenic virus, it can be used as monitor in a large scale study. For MS2 (Paper II) we found that the D-value was always higher than for every virus during all treatment conditions using ammonia, thus, MS2 was a good indicator for all ssRNA viruses tested, and could be used in the large scale assay at the hatchery. Another advantage is that MS2 adsorbs poorly to biosolids, which also was noted in our trials, with a 100% recovery from the HW. Thus it is important to test all viruses in comparison to the bacteriophage in the actual biowaste.

In Paper I, regarding the heat treatments, bacteriophage 28b was evaluated against porcine parvovirus, a suggested monitor virus, and found to be too conservative for this short treatment of 60 min at 70°C. Bacteriophages PRD1 and ϕX174 have proven to be good models for heat inactivation of viruses in several studies. As naturally occurring virus may have a lower inactivation rate of 4 to 10 times than added virus (Lund *et al.*, 1996), ΦX174 as a somatic coliphage has the advantage of being present in biowaste in high amounts.

As bacteriophages are non-pathogenic for humans and animals, and can be propagated to high titres, they are convenient to use in large volumes of biowaste, which is often the case in the field. Further, some bacteriophages, such as f-specific RNA and somatic coliphages can be present endogenously in biowaste, especially in manure, enabling viral reduction measurement over the treatment without any additions. By monitoring bacteriophage reduction at large scale, the survival of the pathogenic viruses or their models could be estimated, in order to reach a safe biowaste management.

Conclusions

- Ammonia treatment of hatchery waste proved to be an effective method for inactivation of ssRNA viruses such as highly and low pathogenic avian influenza of the types studied, and of models for Newcastle disease virus, infectious bronchitis virus and avian hepatitis E virus.
- Based on the inactivation of viruses by different ammonia concentrations, treatment recommendations for outbreak situations regarding these viruses could be given, in order to minimise the risk of viral pathogenic transmission via hatchery waste management.
- The bacteriophage MS2 proved to be an excellent indicator for ssRNA virus inactivation regarding ammonia. Based on MS2 inactivation, treatment recommendations according to EU regulations on ABP can be given, but these are only valid for ssRNA viruses. For ammonia disinfection of hatchery waste to reach a safe end-product that can be used as fertiliser, inactivation of other virus types and parasites should be investigated.
- The thermal process at 70°C for 60 min, simulating a batch process in a biogas plant, did not show a sufficient virus reduction, regarding the thermostable porcine parvovirus. If this virus would serve as model for sufficient virus reduction in thermal treatments regarding Category 3 ABP materials and manure intended for biogas or composting plants, according to EU regulations, it would make demands on other time-temperature combinations very strict. Instead, bacteriophages could be used as indicators, if validated against relevant viruses.

Future research

- Other viruses, such as parvovirus or adenovirus, should also be tested using NH_3 treatment, because double-stranded viruses such as reovirus or rotavirus seem to be less affected by NH_3.
- Since the large-scale trial was performed in May, at temperatures of 19-22°C, additional experiments should be performed during winter, to verify the effect.
- Further investigations regarding survival of AIV in different environmental matrices is needed, in order to assess the risk of environmental transmission to animals and man.

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