Virus Inactivation - Evaluation of Treatment Processes for Food and Biowaste

Eva Emmoth

Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
and
Department of Virology, Immunobiology and Parasitology
Department of Chemistry, Environment and Feed Hygiene
National Veterinary Institute
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2015

Acta Universitatis agriculturae Sueciae
2015:78

ISSN 1652-6880
ISBN (print version) 978-91-576-8354-0
ISBN (electronic version) 978-91-576-8355-7

Print: SLU Service/Repro, Uppsala 2015

Virus Inactivation - Evaluation of Treatment Processes for Food and Biowaste

Abstract

Animal by-products and manure contain valuable plant nutrients that could be recycled onto arable land, as fertiliser. If these materials contain pathogenic microorganisms, such as viruses, transmission to domestic animals, wildlife and the food chain could occur. Virus contamination of food may further occur during all production phases, from slaughter to packaging and distribution. To reduce virus hazards, control measures such as physical and chemical treatments could be applied. As many important food-borne viruses are non-culturable, model viruses are often used to evaluate the effect of virus inactivation methods.

As models for swine hepatitis E virus (HEV) in food treatments, feline calicivirus, murine norovirus and bacteriophages were evaluated. MS2 and ø6 were used as models for highly pathogenic avian influenza virus (HPAIV) in ammonia inactivation and composting of animal by-products, respectively.

In laboratory scale, controlling the factors considered to be the most important for virus inactivation, reduction of relevant and model viruses was assessed as a function of these factors. Recommendations regarding continuously measurable process conditions that should be kept over a certain time to reach sufficient viral reductions could be given, both for normal conditions and in an out-break situation. Bacteriophages could further be used as potential indicators for verification or validation in pilot or full scale processes.

Regimes to assure a 3 $\log_{10}$ reduction for Category 3 materials (2011/142/EC) for ammonia and heat treatment were determined. Further protocols based on pH and temperature to be kept during a certain time for management of HPAIV in outbreak situations were provided based on statistical evaluations of the laboratory results. In high pressure treatment of pork products, pressure and time were defined as critical control points for feline calicivirus and murine norovirus, used as models for HEV.

MS2 and ø6 were successfully used for verification of ammonia treatment and composting, respectively, in larger scale. In food treatments, MS2 was the most conservative indicator of noro and calicivirus inactivation in high pressure and intense light pulse treatments, and øX174 in lactic acid treatments, with potential as models for these types of viruses for verification in production scale.

Keywords: ABP Category 3, AIV, ammonia, bacteriophage, PPV, SVDV, HEV, thermal, high pressure, intense light pulses, lactic acid, virus inactivation

Author's address: Eva Emmoth, Department of Virology, Immunobiology and Parasitology, National Veterinary Institute (SVA), SE-751 89, Uppsala, Sweden
E-mail: eva.emmoth@sva.se

Dedication

To my parents

Contents

List of Publications **7**

Abbreviations **9**

Definitions **10**

1 Introduction **13**

2 Aims of the thesis **17**

3 Background **19**
3.1 General virus properties and inactivation mechanisms 19
3.2 Hazard identification 19
3.2.1 Zoonotic and potentially zoonotic viruses 20
3.2.2 Epizootic viruses 21
3.3 Model viruses 24
3.4 Exposure assessment 24
3.4.1 Soil and manure 25
3.4.2 Water 25
3.4.3 Food matrices 26
3.5 Hazard reducing measures 26
3.5.1 Physical treatments 27
3.5.2 Chemical treatments 31

4 Comments on Materials and Methods **35**
4.1 Choice of model viruses 35
4.2 Design of experiments 39
4.2.1 Virus properties 39
4.2.2 Treatments 39
4.3 Test systems 40
4.3.1 Analysis method 40
4.3.2 Matrix effects 40
4.4 Scale 40
4.5 Statistical evaluations 41
4.5.1 Virus reduction factors (Papers I and IV) 41
4.5.2 Regression analysis (Papers II and III) 41

5 Results and Discussion 43
5.1 Biosafety at normal conditions 43
5.1.1 Thermal treatments (Paper I) 43
5.1.2 Ammonia treatment (Paper II) 44
5.1.3 Critical control points and critical limits 45
5.1.4 Suitability of model viruses and bacteriophages 46
5.2 Food safety (Paper IV) 47
5.2.1 High pressure treatment 47
5.2.2 Lactic acid treatment 48
5.2.3 Intense light pulse treatment 49
5.2.4 Critical control points 50
5.2.5 Suitability of model viruses and bacteriophages 51
5.3 Biosafety in an epizootic outbreak situation (Papers II and III) 52
5.3.1 Critical control points and critical limits 53
5.3.2 Suitability of model viruses and bacteriophages 53
5.4 Managing the risk 54
5.4.1 Considerations for upscaling 55
5.4.2 Epizootic outbreak situation 57

6 Conclusions 59

7 Future perspectives 61

8 Populärvetenskaplig sammanställning 63

References 65

Acknowledgements 81

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. & 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 Emmoth E., Ottoson, J., Albihn, A., Belák S. & Vinnerås, B. (2011). Ammonia disinfection of hatchery waste for elimination of single-stranded RNA viruses. *Applied Environmental Microbiol*ogy 77(12), 3960-6.

III Elving, J., Emmoth, E., Albihn, A., Vinnerås, B. & Ottoson, J. (2012). Composting for avian influenza virus elimination. *Applied Environmental Microbiology* 78(9), 3280-5.

IV Emmoth, E., Rovira, J., Rajkovic, A., Corcuera, E., Wilches, D., Dergel, I., Ottoson, J.R. & Widén, F. Inactivation of viruses and bacteriophages as models for swine hepatitis E virus in food matrices (manuscript).

Papers I-III are reproduced with the permission of the publishers.

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

III Virus part: 10% planning, 30% performance of work, analysis of results, revision of manuscript

IV Virus part: 75% planning, 50% performance of work, 100% analysis and summary of results, 100% writing with revision of co-authors

Abbreviations

ϕX174	*Enterobacteria* phage ϕX174
+ssRNA	Positively single stranded RNA
28B	*Salmonella* Typhimurium phage 28B
ABP	Animal by-products
AHEV	Avian hepatitis E virus
ATCC	American Type Culture Collection
BPIV-3	Bovine parainfluenza virus 3
CCM	Cell culture medium
CCP	Critical control point
CI	Confidence interval
FCoV	Feline coronavirus
FCV	Feline calicivirus
HACCP	Hazard Analysis Critical Control Point
HPAIV	Highly pathogenic avian influenza virus
HW	Hatchery waste
IBV	Infectious bronchitis virus
LPAIV	Low pathogenic avian influenza virus
MNV 1	Murine norovirus 1
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 $Dose_{50}$
UV	Ultraviolet

Definitions

Biowaste	Biological waste
Compost mixture 1	Fresh poultry manure containing 3% straw (wt/wt), used in Paper III
Compost mixture 2	As compost mixture 1 but with addition of 25% (wt/wt) unhatched eggs, used in Paper III
Dose-response assessment	The assessment of the relationship between the degree of exposure (dose) to a virus and the severity and/or frequency of associated disease (response).
D-value	Time required to reduce the number of organisms by 1 $\log_{10}$ (90%)
Exposure assessment	The qualitative and/or quantitative assessment of the expected intake of virus via biowaste or food, including survival in the environment
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 approximately 60%
Hazard	A virus in or associated to biowaste or food which can cause disease in animals or humans.
Hazard characterisation	The qualitative and/or quantitative assessment of the attributes of the disease in animals or humans associated with a virus in or associated to biowaste or food
Hazard identification	The identification of a virus in or associated to biowaste or food which can cause disease in animals or humans.
Matrix	Any environmental condition in conjunction with the virus, such as food and biowaste
Model virus	A virus acting as a model for a pathogenic virus, could also be a bacteriophage
Non-specific model virus	A virus used to verify the robustness of a virus inactivation process, *e.g.* to assure the inactivation of adventitious and emerging viruses, could also be a bacteriophage

Process indicator	A virus or model used to validate the effect of a treatment process
Relevant model virus	The actual pathogenic virus or a virus from the same subgroup
Relevant virus	The actual pathogenic virus or a virus of the same subgroup as the pathogenic virus
Risk assessment	A scientifically based system comprising of: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization.
Specific model virus	A virus from the same family or genus as the pathogenic virus
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
Zoonosis	Disease transmissible between animals and man

1 Introduction

Virus contamination in the food chain may originate from agricultural and urban biological wastes (biowaste). As these materials contain valuable plant nutrients, it is desirable to recycle them by application onto arable land, *e.g.* as fertiliser. However, if biowaste containing pathogenic microorganisms, such as viruses, is applied to farm land, transmission to domestic animals, wildlife and the food chain could occur. Therefore, any pathogen contamination must first be minimised before it reaches environmental reservoirs and water sources (Albihn & Vinneras, 2007). New virus types are constantly emerging, many of them stable enteric viruses (Sun *et al.*, 2015; Belak *et al.*, 2013). New variants of zoonotic viruses, transmissible between animals and man, such as influenza, corona and hepatitis E viruses (HEV) are emerging (Berry *et al.*, 2015; Munoz *et al.*, 2015; Shukla *et al.*, 2011). Moreover, viruses can display strain variability in resistance to inactivation procedures (Li *et al.*, 2013; Terregino *et al.*, 2009; Emerson *et al.*, 2005). Treatment methods must be effective against a range of viruses with different physico-chemical properties, as well as resonable according to economic and practical aspects.

Biowaste includes animal by-products (ABP), defined as "entire bodies or parts of animals, products of animal origin or other products obtained from animals, which are not intended for human consumption" in the European Community regulation (EC, 2009). 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. Category 1 and 2 comprise risk materials that have to be incinerated or sterilised at 133 °C for 20 min at 3 bar or equivalent heat treatment. The lowest risk Category 3 material must be heated to 70 °C for 60 min or equivalent treatment according to EU No. 142/2011 (EC, 2011). Manure belongs to Category 2, but can be used together with Category 3 materials in biogas or composting plants, provided they reach a sufficient pathogen reduction (EC, 2011). Treatment options include

anaerobic digestion, thermal and biological treatments, and disinfection using chemicals such as ammonia (Vinneras, 2007). However, in the food industry, methods for the inactivation of virus must be efficient but at the same time exert minimal adverse effects on the texture and taste of the food. Physical virus inactivation methods such as high pressure and UV treatment using intense light pulses have showed potential in this respect (Kingsley, 2013; Gomez-Lopez *et al*., 2005). Organic acids such as lactic acid act by lowering the pH and are used in food treatments (Rajkovic *et al*., 2010).

Validation of inactivation methods to reduce food safety hazards is a crucial part of the Hazard Analysis Critical Control Point (HACCP) system (CAC, 2003). This system is science based and identifies hazards regarding food safety and any controlled measures (CCP) for their inhibition, elimination or reduction to an acceptable level. Originally aimed for safe food production in the food industry, HACCP has also been used for assessing management strategies for wastewater (Ottoson, 2005) and has potential for biowaste management (EU, 2009). HACCP is recommended as a preventive strategy, as food-borne virus transmission is difficult to prove (FAO/WHO, 2008). This can be due to low virus levels in food, negative impacts of the food matrix, lack of knowledge of virus persistence in foods and poor results of molecular methods in relation to infectiousness and influence on human health (FAO/WHO, 2008).

Moreover, many of the most important food-borne viruses are non-culturable or difficult to culture, such as human norovirus and HEV, an identified zoonotic virus hazard in pork products (Hagiwara, 2007). Good Agricultural Practice (GAP), Good Manufacturing Practice (GMP) and HACCP are recommended for virus control in the food chain (Vasickova *et al.*, 2005). In order to confirm a sufficient virucidal effect of potential CCPs concerning these virus hazards, choosing appropriate process indicators such as model viruses is crucial. In the case of HEV, which is largely uncharacterised, a spectrum of viruses with a range of physico-chemical properties could be appropriate (Sinclair *et al.*, 2011).

The HACCP system is based on seven principles (CAC, 2003):

1. Identification, incidence and severity of hazards, including any microbiological criteria and assessment of control steps.
2. Assessment of the CCPs at the step or steps at which control is crucial to inhibit or eliminate a hazard or reduce it to acceptable levels.
3. Formulation of critical limits at CCPs which separate acceptability from unacceptability.
4. Formulation and implementation of effective monitoring procedures at CCPs.

Principles 5-7 deal with corrective actions when a CCP is not under control, verification procedures and proper documentation of all activities in the HACCP system.

This thesis deals with the use of selected model viruses as process indicators of virus inactivation, as inactivation methods act by different mechanisms. Parts of hazard analysis, identification of CCPs, and validation of critical limits are assessed in two different scenarios. The first of these deals with biowaste and validation of thermal and chemical treatment using ammonia, to achieve biosafety during normal conditions and in an epizootic outbreak situation. The second deals with contaminated food and validation of high pressure, intense light pulse and lactic acid treatments to ensure food safety.

2 Aims of the thesis

The overall aim of this thesis was to minimise the risk of transmission of pathogenic viruses to man and food-producing animals, by evaluating the possibility of using model viruses as indicators of virus inactivation in handling of animal by-products and contaminated food.

Specific objectives were:

- To validate thermal and ammonia treatments on Category 3 animal by-products intended for biogas or composting plants by using parvovirus and enterovirus as *non-specific* and *relevant* model viruses for heat, and to devise a routine method for viral sanitisation of hatchery waste in accordance with current EC regulations.
- To validate ammonia treatment and composting as disinfection methods for poultry animal by-products in the event of an epizootic outbreak by using highly pathogenic avian influenza as a *relevant* virus and low pathogenic influenza, calicivirus, paramyxovirus and coronavirus as *specific* animal model viruses.
- To validate high pressure, intense light pulse and lactic acid as internal and surface treatments of pig food products, using norovirus and calicivirus as *specific* model viruses for hepatitis E virus.
- To investigate the possibility of using bacteriophages as *non-specific* model viruses, indicators of virus reduction in the virus inactivation processes studied.

3 Background

3.1 General virus properties and inactivation mechanisms

Viruses are obligate parasites, *i.e*. require a host cell for replication, and infect all species from bacteria to vertebrates (Anon, 2005). They are composed of single- or double-stranded genetic material (DNA or RNA) enclosed in a protein capsid, sometimes with an outer lipoprotein bilayer, *e.g.* influenza virus. Several of stable enteric viruses spread by the faecal-oral route are food-borne hazards (EFSA, 2011). They commonly possess positive single-stranded RNA, creating secondary structures that confer stability and interaction with molecules such as proteins (Cao & Meng 2012; Weeks, 2010), as seen for HEV (Surjit, Jameel *et al*., 2004), calicivirus (Herbert, Brierley *et al*., 1997) and picornavirus (Verdaguer, Jimenez-Clavero *et al*., 2003; Newman & Brown 1997).

The icosahedral capsid structure confers stability, although susceptibility to virus inactivation could be affected by even single amino acid changes or their accessibility in the protein (Sigstam, Gannon *et al*., 2013; Lima, Vaz *et al*., 2006). A characteristic of importance for susceptibility to inactivation is the nature of the nucleic acid, as the structure of RNA makes it more prone to hydrolytic cleavage than DNA (Soukup & Breaker, 1999; Lindahl, 1993). Virus inactivation mechanisms target the function of attachment and entry to the host cell (capsid) or replication of the virus (nucleic acid and/or any other structural protein active in the replication process).

3.2 Hazard identification

Biowaste permitted for use in biogas or composting plants includes Category 3 ABP materials (EC, 2009) including parts of slaughtered animals fit for human

consumption, hatchery waste and catering waste other than from means of transport operating internationally. These materials should normally be free of pathogens, but there is a risk of *e.g.* pathogenic viruses due to cross-contamination, subclinically infected animals or other residual infectivity (Böhm, 2005). Manure destined for biogas or composting plants could also present a risk due to its pathogenic virus content (Martens & Bohm, 2009). If these virus hazards are not sufficiently reduced before use as *e.g.* fertiliser, they could enter the food chain as a risk for animals and man.

Naked single-stranded DNA viruses such as porcine parvovirus (PPV) (Botner & Belsham, 2012), porcine circovirus 2 (PCV2) (Emmoth, 2005) and chicken infectious anaemia virus (McNulty, 1991) are generally regarded as hazards due to their environmental stability. Enteroviruses prevalent in pig manure (Derbyshire, Monteith *et al*., 1986) are also problematic due to their gastrointestinal infection route, shedding in faeces and great environmental stability (Ley, Higgins *et al*., 2002). Viable avian hepatitis E virus (AHEV) has been demonstrated in chicken eggs (Guo, Zhou *et al*., 2007). For humans, important food-borne viruses are human norovirus, hepatitis A virus, human rotavirus, hepatitis E virus and avian influenza virus (Zuber *et al.*, 2013; EFSA, 2011), the two latter also zoonotic. In general, enteric viruses are transmitted by the feacal-oral route (Fong & Lipp, 2005). Table 1 show some characteristics of excretion of viruses used or discussed in this thesis.

3.2.1 Zoonotic and potentially zoonotic viruses

Of special concern in the enteric virus group is the zoonotic HEV of swine (EFSA, 2011), genotypes 3 and 4 (Van der Poel, 2014), the latter perceived as more pathogenic to humans (Takahashi & Okamoto, 2014). Infection rates of 30-50% in pigs aged 2-4 months have been reported in Sweden and Denmark (Widen *et al.*, 2011; Breum *et al.*, 2010), often with a subclinical course and excreted in faeces and urine. However, HEV primarily replicates in the liver (Feagins *et al.*, 2008), but is also detected in other organs such as spleen, intestines, stomach and kidney, as well as in blood (Okamoto, 2013). HEV in serum has been shown to be associated with lipids and is not neutralised by antibodies that can successfully neutralise faecally excreted HEV (Okamoto, 2013). At slaughter, pigs are about 4-6 months of age and could have an active HEV infection. Food-borne outbreaks have been linked to consumption of raw or undercooked pig liver and pork (Meng, 2011; Yazaki *et al.*, 2003) and cold-smoked pig liver sausage (Colson *et al.*, 2010).

Zoonotic species of avian influenza virus (AIV) are highly pathogenic avian influenza (HPAIV) subtypes H5 and H7, with a mortality rate for humans of

60% for H5N1 (CDC, 2015a). Low pathogenic AIV of subtypes H7 and H9 can also undergo zoonotic spread (CDC, 2015b). If zoonotic influenza contaminates groundwater, it could pose a risk to humans (WHO, 2006). For HPAIV, concerns also exist about gastrointestinal infection through contaminated foods (Vong, 2008). However, the most probable transmission at close range is through bird secretions to the human respiratory mucosa (Brankston, 2007). Potentially zoonotic non-enveloped ssRNA viruses include encephalomyocarditis virus (Brewer *et al.*, 2001; Warren, 1965), and the caliciviruses porcine noro- and sapovirus (Martella *et al.*, 2008; Poel *et al.*, 2000).

3.2.2 Epizootic viruses

Animal viruses of concern for biosafety are epizootic viruses such as HPAIV and Newcastle disease virus (NDV) (Albihn, Nyberg *et al*., 2012). In the event of an epizootic outbreak of HPAIV, poultry farms and hatcheries could be at risk of virus introduction through *e.g.* virus-contaminated eggs, egg trays, lorries or other tools of virus transmission (Swayne, 2008). Large volumes of infected materials such as manure and hatchery waste would then have to be discarded. Both HPAIV and LPAIV H5 and H7, which is classified as "low-pathogenicity notifiable avian influenza" due to its mutation rate in poultry (De Benedictis, Beato *et al*., 2007) are handled as epizootics. Newcastle disease virus is spread by direct contact or contaminated materials, but also by wild birds and the airborne route is up to 60 m (SVA, 2015).

Table 1. *Some characteristics and excretion routes of important pathogenic and zoonotic viruses of the types studied, which can be expected to be present in avian or porcine ABP and raw food, in approximate order according to stability. Adapted from Strauch (1991) and Sattar (2007)*

Virus	Faeces	Organ, body fluid	Egg	Reference
Porcine parvovirus	+++	Uterus, foetus	NA	(Mengeling, 1989)
Swine vesicular disease virus	++ up to 5.7 $\log_{10}$ g^{-1}	Blood, lymph node 2.1-6.2 $\log_{10}$ mL^{-1}	NA	(Sellers, 1982) (Mebus, 1997)
Foot-and-mouth disease virus	++	Skin up to 10 $\log_{10}$ g^{-1}	NA	(Ryan, 2008)
Hepatitis E virus*	+++ up to 7.1 $\log_{10}$ g^{-1}	Swine liver 6.5 $\log_{10}$ $g^{-1;}$ meat; swine carcass up to 5.9 $\log_{10}$ 100 cm^{-2}	NA	(Hagiwara *et al.*, 2007) (Bartolo, 2012) (Jones & Johns, 2012) (Meng, 2010)
Avian hepatitis E virus	+++	Liver, intestines	Yes	(Meng *et al.*, 2008; Guo *et al.*, 2007)
Infectious bronchitis virus	++	Trachea, lungs, kidney	No	(Cavanagh & Gelb Jr, 2008)
Newcastle disease virus	+++	Intestines, trachea	Yes	(Alexander & Senne, 2008) (Capua, 1993) (Chen & Wang, 2002)
Nipah virus*	(+)	Resp. organs, CNS	NA	(Field *et al*., 2007) (Middleton, 2002)
Low pathogenicity avian influenza virus*[a]	+ up to 4.3 $\log_{10}$ mL^{-1}	Resp. secretions up to 5.5 $\log_{10}$ mL^{-1}	Yes	(Cappucci *et al.*, 1985) (Swayne & Halvorson, 2008) (Pillai *et al.*, 2010)
High pathogenicity avian influenza virus*[b]	++ up to 4.5 $\log_{10}$ g^{-1}	Leg muscle 6 $\log_{10}$ g^{-1} Resp. secretions up to 7.7	Yes	(Spickler *et al.*, 2008) (Swayne & Halvorson, 2008)

		log_{10} mL^{-1}		(Koch, 2013)
				(De Benedictis *et al.*, 2007; Chan, 2002)
Porcine influenza virus H1N1pdm09*	(+)	Resp. secretions	NA	(Smith *et al.*, 2009)

NA = not applicable; +++ = primarily excreted in faeces; ++ = clearly excreted in faeces; + = lower incidence; (+) = unlikely to be present in faeces; *zoonotic; [a]*e.g.* subtypes H7 and H9; [b]subtypes H5 and H7

3.3 Model viruses

For risk assessment purposes, the process indicator is chosen according to the identified hazard (Sinclair, 2011). Important properties of a potential model virus include functional morphology, hydrophobicity, genetic stability and prior treatment. For validation purposes, a model virus should preferably be somewhat more resistant than the pathogenic virus in question and easy to detect and cultivate to high titres. In validation of treatment methods, the identified pathogenic virus can often not be used due to personnel biosafety reasons or inadequately equipped laboratories. In commercial production, introducing pathogenic viruses would pose a risk for the consumer (Busta *et al*., 2003).

Virus validation guidelines (EMEA, 1997) exemplify different types of model viruses: If a *relevant* identified virus is present in the material, the actual virus or a virus from the same subgroup should be used. 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 have thus similar physico-chemical properties. In order to determine the robustness of a 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.

Bacteriophages are viruses that infect bacteria and have a similar structure to animal viruses, *i.e*. nucleic acid inside a protein coat. They could thus be considered *non-specific* model viruses, as they exhibit a wide range of physico-chemical properties, with some representatives being enveloped (Adcock, Rice *et al*., 2009). They are suitable virus models because they are harmless to humans, can be grown to high titres and are cheap and easy to analyse. Naturally occurring, they can be defined as *index* (indicating a health risk or pathogenic virus) or *indicator* organisms (indicating the effect of a treatment process or the quality of a particular product) (Havelaar, Butler *et al*., 1991). Another use is as phage cocktails when several pathogenic virus hazards are targeted, choosing bacteriophage groups with the respective similarities (Mesquita & Emelko, 2012).

3.4 Exposure assessment

The aim of exposure assessment in HACCP is to estimate levels of identified hazards at the time of *e.g.* ingestion. If biowaste including ABP is not sufficiently sanitised before being applied as *e.g.* fertiliser, there is a risk of environmental spread. As viruses are not active outside the host and do not cause deterioration of matrices, virus contamination can go unnoticed. In

general, virus persistence in the environment depends on temperature, humidity, pH, microorganism activity, matrix and physico-chemical characteristics of the virus (Hurst, Gerba *et al*., 1980). Thus, some major factors for survival and migration are climate, the nature of the soil (clay content and moisture-holding capacity) and virus type. In an extensive review of enteric virus inactivation by temperature (Bertrand, Schijven *et al*., 2012), norovirus showed D-values of 2-60 days in complex environmental matrices at low temperatures of 0-50 °C.

3.4.1 Soil and manure

Enterovirus survival rates of up to 180 days have been reported for soils (Wekerle, 1986; Ellis & McCalla, 1978), and stability between pH 2 and 9-12, depending on type (Derbyshire, 1989; Herniman, Medhurst *et al*., 1973). Moreover, bacterial biofilm on surfaces is suggested to protect enteric viruses (Vasickova, Pavlik *et al*., 2010). Parvovirus is stable between pH 3 and 10 (Boschetti, Wyss *et al*., 2003; Brown Jr, 1981) and PPV can survive in pig slurry for >43 weeks at 20 °C (Botner & Belsham, 2012), and in soil with minimal reduction for >50 days in a climate chamber (Johansson, Emmoth *et al*., 2005).

Hepatitis E virus has been shown to have a local spread (Widen, Sundqvist *et al*., 2011), and has been found in pig manure pits at 10^3 RNA copies per 60 ml (Kasorndorkbua, Opriessnig *et al*., 2005). It is able to persist in manure for more than 2 weeks, both at 4 °C and at room temperature (USEPA, 2005). Highly pathogenic avian influenza virus and NDV show susceptibility to UVB radiation, with D-values of 160 and 69 min, respectively (Sutton *et al*., 2013), which indicates that solar radiation can inhibit environmental spread. However, pH stability has been shown for subtype H7N9, which can survive for 24 h at pH 4-12 (Zou *et al*., 2013). In chicken manure, LPAIV was found to survive for >20 days at 4 °C (Lu, Castro *et al*., 2003), while it had a D-value of 4-7 days at 20 °C in duck faeces (Nazir, Haumacher *et al*., 2011). Laboratory trials have shown persistence of HPAIV H7N1 for more than one year in manure-amended sandy soil at 5 °C and for 7 weeks at 22 °C (Koch, 2013).

3.4.2 Water

In different groundwater samples at 12 °C, enterovirus has been found to show D-values of approximately 5-29 days (Yates, Gerba *et al*., 1985). Moreover, HEV has been detected in surface waters close to pig holdings (Gentry-Shields, Myers *et al*., 2015) and in fresh food (Maunula, Kaupke *et al*., 2013), for

which irrigation water has been implicated (Brassard, Gagne *et al*., 2012). Indirect transmission of AIV through water is a risk, and D-values for LPAIVs in surface waters of up to 49 days have been reported (Keeler, Dalton *et al*., 2014). Furthermore, LPAIV has been shown to survive longer in wastewater than in lake water (Koch, 2013) and in leachate water for up to 526 days at 4 °C (Graiver, Topliff *et al*., 2009).

3.4.3 Food matrices

As HEV generally has a sub-clinical course in pigs, infected pigs may go to normal slaughter and meat can be contaminated in abattoirs by blood and faeces and this can be passed on to pork products (Bartolo, Diez-Valcarce *et al*., 2012; Berto, Martelli *et al*., 2012). Contact-infected pigs showed HEV RNA in muscle samples, which was attributed to infectious blood (Bouwknegt, Rutjes *et al*., 2009). The persistence of HEV in pork products is estimated as high, as reviewed by Cook and van der Poel (2015), *e.g.* only a 0.45 $\log_{10}$ reduction in HEV gt3 after 50 days at 22 °C in liver suspension and an even lower reduction at 4 °C. Heating to 71 °C for 20 min is recommended for complete inactivation (Barnaud, Rogee *et al*., 2012).

Norovirus surrogates survive better on ham than on inert surfaces (Mattison *et al.*, 2007). Regarding other food-borne viruses, human norovirus (HuNoV) and the surrogate murine norovirus (MNV) have been detected for >70 days at 7 °C dried on surfaces (Mormann, Heißenberg *et al*., 2015) and it has been seen that they can be internalised in lettuce via roots (DiCaprio, Culbertson *et al.*, 2015), depending on virus type. Moreover, LPAIV and HPAIV H7 strains have been reported to stay infectious on poultry meat for 200 and >50 days at 4 and 20 °C, respectively (Beato, Mancin *et al*., 2012).

3.5 Hazard reducing measures

A previous evaluation of methods to reduce viral hazards in sludge suggested a criterion of 4 $\log_{10}$ reduction based on estimation of enterovirus content (Carrington, 2001). However, more recent regulations concerning ABP (EC, 2009) state that Category 3 material and manure destined for biogas or composting plants must be heated to 70 °C for 60 min, or equivalent treatment according to EC No. 142/2011 (EC, 2011). The latter regulation states that a validated thermal or chemical process can be used for these materials, provided it gives a reduction of at least 3 $\log_{10}$ of a thermo-resistant virus, whenever it is

deemed a risk. To ensure inactivation of food-borne viruses, heat at ≥90 °C and HPP treatment are reported to be the most effective (Zuber *et al.*, 2013)

3.5.1 Physical treatments

Thermal treatments

Thermal treatment to reduce virus load is a common strategy in food industries and for biowaste (Bertrand *et al.*, 2012). For the latter, heat can be applied by different means, *e.g.* 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 (Albihn & Vinneras, 2007). Any biosolids present can even retain heat and result in faster virus inactivation (Bertrand, Schijven *et al.*, 2012; Turner & Burton, 1997). An exception is when the virus is embedded in tissue, as this has been shown to protect viruses (Lund *et al.*, 1996; Dimopollous *et al.*, 1959). Table 2 shows some inactivation rates of the types of viruses used in this thesis.

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, Schmidt *et al.*, 2002), suggesting that different capsid structures influence the susceptibility to heat. Regarding MNV, in a study by Hirneisen and Kniel (2012) heating at 80 °C for 5 min gave complete reduction and also denatured the proteins, in contrast to UV radiation and high pressure processing (HPP). For bacteriophage MS2, heating at 72 °C affected only the virus protein's ability to bind to host cells, rendering the virus non-infectious (Wigginton, Pecson *et al.*, 2012). Specific inhibitors of heat inactivation include anionic detergents, collagen and cystin, while volatile fatty acids and NH_3 increase inactivation (Popat, Yates *et al.*, 2010), and the authors quote that regarding enteroviruses, heat acts on both the nucleic acid and the capsid protein, with a shift to protein denaturation at about 50 °C for poliovirus. Lowering the ionic strength stabilises DNA viruses such as parvovirus, while the opposite effect occurs for enteroviruses (Wigand, Bachmann *et al.*, 1981). Thus, the character of the different matrices 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, Shannon *et al.*, 1986) than during ordinary heat treatment of SVDV, another enterovirus, at 56°C for 60 min (Herniman, Medhurst *et al.*, 1973) (Table 2).

Table 2. *Physical treatments (thermal, high pressure and intense light pulses) shown to reduce viruses in different biowaste materials or food, as discussed in this thesis (adapted from (Zuber et al., 2013)*

Virus	Temperature	Material	D-value[a]	Reference
Avian influenza virus	35°C	Compost run-off	6.3	(Guan *et al.*, 2009)
	35°C	Pre-compost water	23.5	
High pathogenic avian influenza virus	37°C	Allantois fluid	60	(Terregino *et al.*, 2009)
High pathogenic avian influenza virus	55°C	Whole egg	0.17	(Swayne *et al.*, 2004)
Porcine parvovirus	55°C	Biogas substrate[b]	14	(Lund *et al.*, 1996)
Porcine parvovirus	55°C	Fresh manure	11	(Elving *et al.*, 2014)
Porcine parvovirus	55°C	Biogas substrate	4.8	(Holmqvist *et al.*, Manuscript)
Hepatitis E virus	56°C	Faecal suspension	~0.3	(Emerson, 2005)
Bovine enterovirus	55°C	Cattle manure[b]	≤0.07	(Monteith *et al.*, 1986)
Swine vesicular disease virus	56°C	Pig slurry	0.4	(Herniman *et al.*, 1973)
	60°C		≤0.03	
Swine vesicular disease virus	65°C	Pig slurry	~0.005	(Turner & Williams, 1999)
Porcine parvovirus	70°C	Manure/ household waste	0.7	(Lund *et al.*, 1996)
Bovine parvovirus	70°C	Slurry	0.4	(Böhm, 2005)
Porcine parvovirus	70°C	Biogas substrate	0.75	(Holmqvist *et al.*, manuscript)
Hepatitis E virus	71°C	Swine liver	~0.04	(Barnaud *et al.*, 2012)

Virus	Pressure/ temperature/time	Material	Reduction log_{10}	Reference
Bacteriophage MS2	500 MPa/20°C/10 min	Oyster slurry	0.22	(Black *et al.*, 2010)
Feline calicivirus	200 MPa/5°C/5 min	Cod	1.15	(Hirneisen *et al.*, 2012)
Murine norovirus	500 MPa/20°C/2 min	Green onion	4.7	(Hirneisen & Kniel, 2013)
Human norovirus	600 MPa/6°C/5 min	Oysters	4 (PCR)[c]	(Leon *et al.*, 2011)
Murine norovirus	400 MPa/5°C/5min	Oysters	4	(Kingsley *et al.*, 2007)
Feline calicivirus	500 MPa/4-13°C/5 min	Sausage	2.9	(Sharma *et al.*, 2008)
Bacteriophage øX174	500 MPa/4-13°C/5 min	Sausage	1.6	(Sharma *et al.*, 2008)
Human norovirus	600 MPa/1°C/2 min	Dry blueberries	0.5 (PGM[d]/PCR)	(Li *et al.*, 2013)
Avian influenza virus	500MPa/ 15°C/25 s	Chicken meat	>5	(Isbarn *et al.*, 2007)
Virus	ILP dose (J cm^{-2})	Material	Reduction log_{10}	Reference
Polio	2	PBS 5%v/v FCS	5.5	(Roberts & Hope, 2003)
Encephalomyocarditis virus	2	PBS 5%v/v FCS	>6.1	(Roberts & Hope, 2003)
Hepatitis A virus	2	PBS 5%v/v FCS	>5.6	(Roberts & Hope, 2003)
Parvovirus	2	PBS 5%v/v FCS	2.4 - >6.4	(Roberts & Hope, 2003)
Murine norovirus	0.146	PBS 5%v/v FBS	4.1	(Jean *et al.*, 2011)
Hepatitis A virus	0.146	PBS 5%v/v FBS	2.1	(Jean *et al.*, 2011)
Murine norovirus	8.98	Stainless steel, fouled	2.6	(Vimont *et al.*, 2015)
Bacteriophage MS2	28.2	Powdered black pepper	0.61	(Belliot *et al.*, 2013)

[a]D-value, i.e. time (h) to reduce the virus titre by 1 $log_{10,}$ as related or approximated from the data; [b]during anaerobic digestion; [c]analysed by polymerase chain reaction; [d]analysed in combination with porcine gastric mucin attachment

High pressure processing

High pressure processing is an emerging strategy for food preservation and has been shown to reduce virus contamination in food such as shellfish and sausage (Table 3). This method has been shown to irreversibly inactivate viruses even below 250 MPa, affecting the non-covalent associations of the biomolecules, both for enveloped (Isbarn, Buckow *et al*., 2007; Jurkiewicz, Villas-Boas *et al*., 1995; Silva, Luan *et al*., 1992) and naked (Pontes, Cordeiro *et al*., 2001; Silva & Weber, 1988) RNA viruses.

It has been shown that HPP affects viral capsid proteins of MNV (Hirneisen & Kniel, 2012; Tang, Li *et al*., 2010), retaining antigenicity. Low temperatures increase inactivation of feline calicivirus (FCV) (Chen, Hoover *et al*., 2005) and norovirus (Leon, Kingsley *et al*., 2011; Kingsley, Holliman *et al*. 2007). Salt hinders the effect for FCV and MNV and acidic conditions decrease inactivation for MNV (Kingsley, 2013). Interestingly, picornaviruses have differing susceptibility to HPP, presumably depending on structural differences in receptor-binding domains (Kingsley, 2013). Table 2 shows some examples of HPP treatments.

Intense light pulses

Intense light pulse (ILP) treatment is a novel food technology used for surface decontamination, consisting of broad-spectrum light, rich in UV, delivering short pulses with high energy (Gomez-Lopez, Ragaert *et al*., 2007). Inactivation of enteric viruses of up to 5 $\log_{10}$ has been seen in solutions (Jean *et al.*, 2011; Roberts & Hope, 2003) and of approximately 4 $\log_{10}$ MNV 1 on surfaces (Vimont *et al.*, 2015).

Intense light pulses break RNA and capsid proteins of MNV 1, an effect suggested to be caused by absorbance of ultraviolet light followed by instantaneous overheating transferred to water (Vimont, Fliss *et al*., 2015). Bacteriophage MS2 shows capsid degradation after ILP treatment preceding the effects on the genome (Belliot, Loutreul *et al*., 2013). The capsid structure could influence susceptibility to UV inactivation due to amino acid differences, as shown for f-specific RNA phages (Wigginton, Menin *et al*., 2012).

The food matrix affects reduction, hindering access by ultraviolet light (Gomez-Lopez, Ragaert *et al*., 2007). A high protein or fat content can hinder the ILP effect, as can the ability of different food surfaces to reflect and absorb light (Gomez-Lopez, Devlieghere *et al*., 2005), thus leaving less energy for

inactivation of microorganisms. Table 2 shows some examples of ILP treatments.

3.5.2 Chemical treatments

Ammonia treatment

Ammonia (NH_3) in the form of ammonium salts is a common fertiliser. However, NH_3 in its 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. The equilibrium between NH_3 and its ionised form, NH_4+, depends on the pH and the temperature and is shifted toward NH_3 when either of these increases (Emerson, 1975). A common compound in biowastes, such as wastewater sludge (Ward & Ashley, 1977) and manure (Hansen *et al.*, 1998), ammonia has been evaluated for sanitation purposes both by natural routes (Vinneras *et al.*, 2008) and in added form (Vinnerås *et al.*, 2003).

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 a pH increase inside the virus particle promoting alkaline hydrolysis, resulting in degradation of the viral RNA (Ward, 1978). The reaction is irreversible, but reovirus, a dsRNA virus, has been shown to be more resistant (Ward & Ashley, 1977), as is the dsDNA-containing phage 28b (Vinnerås *et al.*, 2008; Höglund *et al.*, 2002).

The inactivation mechanism by ammonia was further elucidated by Decrey *et al.* (2015), who found for the ssRNA bacteriophage MS2 that general alkaline transesterification of RNA was responsible and equally effective on naked RNA. The theory was that the 2’ carbon of the sugar ribose in RNA contains a hydroxyl group close to the centre of the phosphodiester bond. In a basic environment, the hydroxyl group is deprotonated, rendering the oxygen more nucleophilic for attack against the phosphorus centre of the phosphodiester bond, with subsequent scission. Decrey *et al.* (2015) observed varying inactivation rates for the ssRNA viruses MS2, GA and Echovirus, which they attributed to the different folding of the respective RNA structure, allowing more or less in-line structures promoting transesterification. Moreover, a longer genome gives more opportunity for formation of an in-line

structure and thus Echovirus can be reduced faster than MS2 and GA, which have shorter genomes.

Concerning the slower inactivation rate of the dsRNA virus, this is suggested to be due to less in-line structure, owing to conformation restrictions of the double helix. Alternatively, it could be due to the fact that two scissions are needed within a certain length to cleave the strands (Burge *et al.*, 1983). As for the DNA in DNA viruses, this molecule does not possess any hydroxyl group on the 2' carbon and thus cannot be cleaved by this transesterification mechanism, although cleavage of peptide bonds is enhanced at higher pH (Decrey, 2015). Table 3 shows results from different ammonia studies.

Lactic acid treatment

For food preserving purposes, lactic, acetic and citric acid are the most commonly used organic acids (Rajkovic *et al.*, 2010). The virus reducing properties of lactic acid have been evaluated both naturally in fermented sausage products and in added form (Straube *et al.*, 2011; Straube *et al.*, 2010). In biowaste, lactic acid has been shown to inactivate picornavirus (Scheinemann *et al.*, 2015) and as part of acidification of chicken pulp it completely inactivates LPAIV H5N2 (Kabell *et al.*, 2009). Other organic acids used in biowaste management include formic acid, which is used to stabilise ABP 1 aimed for combustion (Vinneras *et al.*, 2012)

The mechanism of inactivation of acetic acid and benzoic acid is mediated by the undissociated molecule (Jeffrey, 1995), and only certain ion forms of ascorbic acid may be active against enteroviruses (Salo & Cliver, 1978). The virucidal action of organic acids on enveloped viruses is due to interaction of lipophilic structures with the viral lipid membranes (Haas *et al.*, 1995). Other effects include inhibition of enzymatic reactions (De Benedictis *et al.*, 2007) and, for ascorbic acid, breakage of DNA, as seen for the double-stranded phage T7 (Richter & Loewen, 1982). For naked viruses, the virucidal action of organic acids is suggested to have an inhibiting effect on nucleic acid and capsid proteins, the latter hindering virus adsorption onto host cells (Choi, Song *et al.*, 2010; Salo & Cliver, 1978). The action seems to be virus specific, with diverging effects of lactic acid on FCV and Echovirus (Straube, Albert *et al.*, 2011) and of ascorbic acid on different enteroviruses (Salo & Cliver, 1978). Moreover, the effect is not only a result of pH, as poliovirus is significantly more reduced by ascorbic acid than citric acid at the same pH. Salo and Cliver (1978) claim that serum decreases the rate of inactivation of ascorbic acid, in accordance with the fact that protein content inhibits the effect of organic acids (Haas *et al.*, 1995). Other factors affecting food treatments

with organic acid are the type, pH and buffering capacity of the product (Rajkovic, Smigic *et al*., 2010).

Table 3. *Chemical (ammonia and organic acid) treatments of viruses discussed in this thesis*

Virus	Chemical/concentration	Time/pH/temp	Material	D-value[a]/Reduction log_{10}	Reference
AIV H3N8	Lactic acid/0.20%	1h/3.8-3.9/4°C	PBS lactic acid	n.a./~6 log_{10}	(Straube *et al.*, 2010)
Feline calicivirus	Lactic acid/0.4%	3h/3.2-3.3/20°C	PBS lactic acid	n.a./~1.5 log_{10}	(Straube *et al.*, 2011)
Porcine parvovirus	Formic acid/3%	60d/4.5/14°C	Carcasses, slaughterhouse waste	n.a./6 log_{10}	(Vinneras *et al.*, 2012)
Polio virus, Coxsackie virus, Echovirus	Ammonia/294 mM	24h/9.5/21°C	0.1 M Tris-0.5 M NH_4Cl	<4/>6	(Ward & Ashley, 1977)
Bacteriophage f2 (*Leviviridae*)	Ammonia/36 mM	150h/8.6/20°C	0.25 M NH_4Cl	14/n.a.	(Cramer *et al.*, 1983)
Bacteriophage MS2	Ammonia/85 mM	7d/8.9/15°C	Urine	<19/8.9	(Chandran *et al.*, 2009)
Bacteriophage MS2	Ammonia/156 mM	182d/9.0/24°C	Urine	15±3/n.a.	(Vinneras *et al.*, 2008)
Reovirus 3	Ammonia/294 mM	24h/9.5/21°C	0.1 M Tris-0.5 M NH_4Cl	>12/<2	(Ward & Ashley, 1977)
Rhesus rotavirus	Ammonia/66 mM	~120d/9.0/20°C	Urine	35d/>3 log_{10}	(Höglund *et al.*, 2002)

[a]D-value= time (h) to reduce the titre by 1 log_{10} as reported or approximated from the data; n.a.=not applicable

4 Comments on Materials and Methods

Guidelines have been issued on the design and interpretation of virus validation studies (EMEA, 1997; EMEA, 1996), due to the hazard of unknown or adventitious viruses reported to contaminate biological products such as vaccines and blood products in the past (EMEA, 1996). These guidelines give practical and statistical advice, such as types of model virus (sections 3.3 and 4.1) and validation of test systems (section 4.2), which are important for retrieving reliable data. For full details of the materials and methods used, see the relevant sections in Papers I-IV.

4.1 Choice of model viruses

In HACCP, validation of inactivation processes are performed using suitable viruses or models, depending on the virus hazard identified. Table 4 shows the model viruses and bacteriophages used in this thesis.

In Paper I, PPV was chosen as *non-specific* model virus to validate a thermal step stipulated by the ABP regulation (EU, 2009), also a *relevant* virus. Swine vesicular disease virus, an enterovirus, which has been suggested as a model for processes in biogas plants (Lund, Jensen *et al*., 1996), was chosen as a *relevant* and *specific* model virus. An additional *non-specific* model, bacteriophage 28b, validated for large-scale possibilities and previously used as a model in mesophilic composting of household waste (Holmqvist & Stenström, 2002), was also included.

In Paper II, evaluating ammonia treatment of hatchery waste, LPAIV H5N3 and HPAIV H7N1 were chosen as *relevant* viruses, as they could be present in hatchery waste following an epizootic outbreak. The *specific* model viruses used in Paper II were bovine parainfluenza virus (BPIV) 3, a model for NDV, and feline coronavirus (FCoV), a model for IBV. According to the definitions,

feline calicivirus (FCV) would be considered a non-specific model virus for AHEV, but could be suggested as a *specific* model virus, as AHEV was formerly in the same virus family, the Caliciviridae. Based on its similarity to the pathogenic viruses, bacteriophage MS2 was used in Paper II as a *non-specific* model, also to be evaluated as a stable model regarding alternative processes according to ABP regulations (EU, 2011).

In Paper III, evaluating composting of hatchery waste and manure as a means of inactivation of epizootic avian influenza, HPAIV H7N1 was chosen as a *relevant* virus. Based on its similarity to the pathogenic virus, the enveloped bacteriophage ϕ6was used as a *non-specific* model. Bacteriophage MS2 was included as another *non-specific* model, but could also be a model for other heat-stable avian viruses such as AHEV.

In Paper IV, evaluating food processing technologies using contaminated swine liver, ham and dry sausage, a special case arose as HEV is very difficult to cultivate and susceptibility is estimated from models using different physico-chemical properties (Sinclair, Rose *et al*., 2012). The *specific* model viruses were FCV and MNV, as models for HEV and other food-borne human viruses. According to the definitions, FCV and MNV would be considered *non-specific* model viruses for HEV, but could be suggested as a *specific* model virus, as HEV was formerly in the same virus family, the *Caliciviridae*, and thus possesses similar physico-chemical characteristics. Bacteriophages MS2, øX174 and 28b were used as *non-specific* model viruses with diverse structures.

Table 4. *Properties of the virus/bacteriophage families of the viruses used in this thesis (Anon, 2005) compared with swine hepatitis E*

Virus/bacteriophage	Shape	Virus family	Size (nm)	Envelope	Genome	
					Type	Size (kb)
Porcine parvovirus[a] (I)	Icosahedral	Parvoviridae	18-26	No	ssDNA	4-6
Swine vesicular disease virus[b] (I)	Icosahedral	Picornaviridae	~30	No	+ssRNA	7-8.8
Avian influenza virus[c] (II,III)	Spherical/ pleomorphic	Orthomyxoviridae	80-120	Yes	Segmented - ssRNA	10.0-14.6
Bovine parainfluenza virus 3[d] (II)	Spherical/ pleomorphic	Paramyxoviridae	~150	Yes	-ssRNA	15-18.4
Hepatitis E virus	*Icosahedral*	*Hepeviridae*	*27-34*	*No*	*+ssRNA*	*7.2*
Feline coronavirus[e] (II)	Spherical	Coronaviridae	120-160	Yes	+ssRNA	27.6-31
Feline calicivirus[f] (II,IV)	Icosahedral	Caliciviridae	27-40	No	+ssRNA	7.4-8.3
Murine norovirus[g] (IV)	Icosahedral	Caliciviridae	27-40	No	+ssRNA	7.6
Enterobacteria phage MS2[h] (II)	Spherical/ icosahedral	Leviviridae	26	No	+ssRNA	3.5-4.3
Enterobacteria phage ϕX174[i] (IV)	Icosahedral	Microviridae	~30	No	circular +ssDNA	4-6
Salmonella Typhimurium phage 28B [j](I,IV)	Isometric icosahedral with tail	Podoviridae	60-65	No	dsDNA	38-42
Pseudomonas phage ϕ6 (III)	Icosahedral nucleocapsid	Cystoviridae	85	Yes	dsRNA, segmented	13.4

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) and wildtype/84 (SVA); [g] strain SVA/07 [h]ATCC 15597-B1; [i]ATCC 13706-B1; [j](Lilleengen, 1948); [k]ATCC 21781-B1

4.2 Design of experiments

4.2.1 Virus properties

The viruses used in Papers I-IV were naturally infected to high titres, using their respective cell line, with no further manipulation other than low-speed centrifugation to avoid aggregation. Aggregation could otherwise increase physical removal and decrease inactivation, and thus alter the correlation with the actual process step (EMEA, 1997). The viruses were spiked in such a way (≤10%) as not to significantly affect the properties of the different matrices. In the experiments with solid food matrices (Paper IV), the virus spike was up to 15%, so as to cover the surface of the food item.

4.2.2 Treatments

The reproducibility of an effective step should be assessed by performing at least two independent experiments (EMEA, 1997). In all laboratory studies in Papers I-IV, at least two experiments were performed on different occasions. Three different batches of substrate from a biogas plant were used in thermal studies (Paper I). In the ammonia studies (Paper II), HW originating from two large batches of 1-2 m^3 with a pH of 8.0 and 7.4, respectively, was used. Dry matter content was 60% for the HW in the laboratory study. In the composting study (Paper III), the poultry manure originated from two batches, added with unhatched spf-eggs containing 14-day old embryos from one batch and straw from one batch, with a moisture content of 57-58% for the two compost mixtures. For Paper (IV), the food items originated from different batches for the three treatment methods HPP, LA and ILP.

In addition to the minimum exposure time, at least one time point less than the minimum exposure time and exceeding zero is recommended for virus inactivation studies (EMEA, 1997). All experiments where time was monitored fulfilled this criteria (Papers I-IV). The amount of virus eliminated or inactivated by the treatment should be compared to the amount of virus that may be present in the materials (EMEA, 1997). In Papers I-IV, the virus amount varied from approximately 5-8 $\log_{10} g^{-1}$, usually with the higher titres for the bacteriophages. Regarding biowaste destined for biogas or composting plants, it is difficult to estimate the exact virus content, but animal faeces, organs and body fluids can contain pathogenic virus in large amounts (Table 1).

4.3 Test systems

4.3.1 Analysis method

The evaluation methods were based on the infectivity of the virus using their relevant detection system, in this case a susceptible cell line showing cytopathogenic effect. As PPV is non-cytopathogenic, the infection was combined with an immunological method. Titre calculations were based on quantal methods for the viruses (Kärber, 1931) and quantitative methods for the bacteriophages (Anon, 2000; Anon, 1995; Adams, 1959).

The titre values obtained reflect the sensitivity of the detection system to the virus/bacteriophage. Not all living virus particles are necessarily detected (Havelaar *et al.*, 1991), and thus virus reduction factor (RF) or D-values were calculated to estimate the efficiency of the inactivation process.

4.3.2 Matrix effects

Any toxic or interfering effects of the matrices have to be considered regarding the reliability of the detection system (EMEA, 1997). Cytotoxicity and virus interference assays were performed in order to determine the effect of the different matrices on the sensitivity of their detection systems. 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 volume tested and c is the virus concentration (EMEA, 1997)

4.4 Scale

The process factors at laboratory or pilot plant scale could differ from those of commercial-scale processing, despite efforts to design the scaled-down process (EMEA, 1997). Thus, variations in process parameters have to be evaluated in terms of effect on virus inactivation (EMEA, 1997). This concept was used in the present thesis, as the response variable (change in virus titres) was measured in response to controlled parameters.

Based on the results from the statistical evaluations (see section 4.5), upscaling of the ammonia treatment was performed in an actual production setting at a hatchery (Paper II). Bacteriophage MS2 was mixed in a total volume of approximately 2 m^3 HW, and NH_3 was introduced at a slow rate to

up to 0.6%(w/w) with sampling from both the top and bottom of the tank (Paper II). MS2 titre, environmental temperature, NH_3 concentration and pH were monitored. In composting (Paper III), duplicate laboratory-scale compost reactors were used for upscaling and surface temperature, moisture content (%), pH and carbon/nitrogen ratio were monitored.

4.5 Statistical evaluations

4.5.1 Virus reduction factors (Papers I and IV)

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 (EMEA, 1997) 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. It is recommended that the 95% confidence limits for the reduction factor are calculated wherever possible in studies of clearance of relevant and specific model viruses (EMEA, 1997). The relevant reduction factors with 95% confidence interval in Paper I and Paper IV are presented in this thesis (EMEA, 1997).

4.5.2 Regression analysis (Papers II and III)

For each microorganism, ammonia 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} was extrapolated using the upper 95% confidence interval. For Paper III, the 99% confidence intervals (99% CI) were calculated only for material and temperature combinations with data available from at least three sampling occasions, so in this case the time for a 12-log_{10} reduction in the organisms was based on the upper 99% CI.

5 Results and Discussion

5.1 Biosafety at normal conditions

5.1.1 Thermal treatments (Paper I)

The results from the thermal treatments regarding SVDV and PPV in biogas substrates are shown in Table 5. At 70 °C, SVDV underwent complete reduction after 30 min, comparable to the reduction of about 6.5 $\log_{10}$ reported at 65 °C for 2 min in pig slurry (Turner *et al.*, 1999). In contrast, at 55 °C SVDV showed lower reductions, with an estimated D-value of 0.5 h. These results are in good agreement with previous findings for pig slurry at 56 °C of an estimated D-value of 0.4 h (Herniman *et al.*, 1973). At higher temperatures, those authors found complete reduction of ≥6.5 $\log_{10}$ at 60 °C after 10 min, and noted that SVDV was more stable in slurry than in milk, probably due to matrix differences.

For PPV at 55 °C, we obtained no significant inactivation. For longer time periods, Lund *et al*. (1996), who tested PPV at 55 °C in laboratory biogas reactors fed manure/manure with 20% household waste (Table 2), observed biphasic inactivation curves, with D-values of 3 and 14 h, respectively. Similarly, (Elving *et al.*, 2014) tested PPV in fresh cattle manure and obtained a D-value of approximately 11 h. Both these studies used 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*. (2014) had a dry matter content of about 13%. This shows the conservative nature of PPV towards heat.

For PPV at 70 °C we found an estimated D-value of 0.3 h. This is in agreement with (Böhm, 2005), who reported a D-value of 0.4 h for bovine parvovirus in slurry (Table 2). However, D-values of 0.7-0.75 h have been found for PPV in biogas substrates (Holmqvist *et al.*, Manuscript; Lund *et al.*, 1996). The latter opposed the requirement of a 4 $\log_{10}$ reduction set by

(Carrington, 2001) as being too high for such a heat-resistant virus as PPV. Instead, they recommended the use of a picornavirus.

Table 5. *Reductions ($TCID_{50}$ log_{10} ±95%CI) brought about by thermal treatments (Paper I). Values are from triplicate experiments, for original values see Paper I*

Virus	55 °C 30 min	55 °C 60 min	70 °C 30 min	70 °C 60 min
PPV	1.0±1.4	1.4±1.4	2.6±1.5	3.2±1.1
SVDV	1.5±0.53	2.2±0.47	≥6.0[a]±0.85[b]	≥5.9[a]±0.85[b]

[a]Titre fell below detection limit; [b]95%CI of initial titre

5.1.2 Ammonia treatment (Paper II)

All inactivation rates increased significantly with NH_3 addition and temperature. The enveloped viruses, not tested previously in terms of NH_3 treatment, had similar reduction rates as the naked viruses. Bacteriophage MS2 had consistently higher D-values than the pathogenic viruses and models, as shown at 0.25% (wt/wt) ammonia addition at 14 °C (Table 6).

Table 6. *Effect of ammonia addition 0.25% (wt/wt) to hatchery waste, pH 9.2, NH_3 concentration 74 mmol kg^{-1}, at 14°C (Paper II).*

Virus/phage	Inactivation rate ±SE (log h^{-1})	D-value (95%CI) (h)	Time (h) to 3 log_{10} reduction
FCoV	0.39±0.077	2.6(1.8-4.7)	14
BPIV 3	0.29±0.04	3.4(2.6-5.0)	15
FCV	0.41±0.032	2.4(2.1-2.9)	9
LPAIV	0.31±0.031	3.2(2.6-4.2)	12
HPAIV	0.25±0.027	4.1(3.3-5.4)	16
MS2	0.069±0.0036	14(13-116)	49
MS2*	1.3	0.77	2.3

*Full-scale experiment up to 214 mmol kg^{-1}, pH 10, 22°C; n.a. = not applicable

The results in Paper II regarding the naked ssRNA viruses are in good agreement with earlier reports studying NH_3 inactivation. In the treatment 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 to be Picornaviruses, showed a D-value of 1.3 h. In similar conditions, (Ward & Ashley, 1977) showed that at 21 °C, pH 9.5 and an NH_3 concentration of 294 mM, six different Picornaviruses were inactivated by >6 log_{10} in 24 h (D-value <4 h).

In the full-scale experiment at a hatchery (Table 6), MS2 was inactivated with a D-value of 0.77 h, *i.e.* a three-fold greater reduction in the full-scale

process compared with that in the present bench-scale process at similar pH values and NH_3 concentrations.

5.1.3 Critical control points and critical limits

For HACCP purposes, in the European Community regulation (EC, 2009) the hazards are already identified and generally characterised into categories (1, 2 and 3). For the Category 3 materials used in Papers I and II, regulation EU No. 142/2011 (EC, 2011) states that a thermal or a chemical process may be used provided it gives a reduction of at least 3 $\log_{10}$ in a thermo-resistant virus, whenever it is deemed a risk.

Swine vesicular disease virus, a model for enterovirus, reached reductions of ≥6.0±0.85 (95%CI) $\log_{10}$ already after 30 min at 70 °C in Paper I. Thus the temperature and time in the batch heating process evaluated in that study could be suggested as CCPs in the treatment of this mixed biogas substrate, also for virus groups of similar susceptibility. However, for PPV at 70 °C there were lower mean reductions of 2.6±1.5 (95%CI) $\log_{10}$ and 3.2±1.1 (95%CI) $\log_{10}$ after 30 and 60 min, respectively, indicating that other heat-stable viruses such as circoviruses would also survive these high temperatures. 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). Thus, depending on the individual hazard analysis by the biogas or composting plants, CCPs have to be evaluated on a case-by-case basis.

In the validation of the 70 °C heating step for 1 h, the process mimicked the batch process in a biogas plant. The experiments were performed with continuous stirring at 70.0-70.7 °C, and a pre-heating time of 20 min before time-keeping started at 70 °C. Thus, a critical temperature limit could be suggested as 71 °C. For enterovirus, the critical time limit could be decreased to 30 min in addition to the pre-heating time. The temperature has to be measured, preferably continuously, with well-calibrated thermometers. The stirring process is crucial to achieving an even temperature distribution.

Based on the results of ammonia treatment in hatchery waste, an addition of 0.25-0.75% (wt/wt) could be used for ssRNA virus inactivation in hatchery waste, at temperatures of 5-25 °C for all viruses tested. The CCPs would be pH and temperature, and treatment times could be devised based on the MS2 D-values with their 95% confidence intervals (Table 6). To achieve the intended target of a 3 $\log_{10}$ reduction (EC, 2011), *e.g.* addition of 0.25% (w/w) NH_3 (10

L 28% NH_3 [aq] m^{-3} material) should be followed by storage for 2 days at 14 °C (Table 6). In this case, the critical limits are a temperature of >14°C, a pH of >9.2 and a time of >2 days.

5.1.4 Suitability of model viruses and bacteriophages

Thermal treatment

The aim of the thermal treatment according to EU regulations (EC, 2011) is to inactivate also adventitious virus hazards. Thus, parvovirus was chosen, as a thermostable virus, and an enterovirus model. Considering the inactivation mechanism (see section 3.5.1), at the high temperatures of 55-70°C, the different effects obtained could be due to differing protein structures. The dsDNA bacteriophage 28b used as a presumptive indicator 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. In a previous study, 28b was found to be six- and seven-fold more heat resistant than PPV at 55 °C and 60 °C, respectively (Holmqvist *et al.*, manuscript), showing promise as an indicator for longer treatment times for an identified viral hazard.

Ammonia treatment

In preliminary studies, bacteriophage ϕX174 did not show any significant inactivation at 25 °C up to 72 h even at 0.75% (w/w) NH_3, with an estimated D-value of approximately 25 days at 0.25% (w/w) NH_3 at pH 9.2. This is in the same range as found by others (Vinneras *et al.*, 2008), *i.e.* a D-value of 12±10 days at an NH_3 concentration of 156 mM NH_3, 24 °C and pH 9.0. These results confirm that bacteriophage ϕX174 is very insensitive to NH_3 inactivation, as indicated by the proposed inactivation mechanism (see section 3.5.2), and is thus too conservative as an indicator for ssRNA viruses in these conditions.

In contrast, bacteriophage MS2 proved an excellent indicator of inactivation of all other ssRNA viruses, as it was always inactivated at a lower rate. Similarly, Cramer *et al.* (1983) assessed the effect of NH_3 on bacteriophage f2, which is in the same family (the Leviviridae) as MS2, and observed 4.5-fold 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, they 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. Bacteriophage f2 has also been used in experiments with non-aerated animal waste, where the effect of NH_3 was noted at 16 °C and pH 8.7 (Pesaro *et al.*, 1995). 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.

The viruses used in the ammonia treatments were all ssRNA viruses, models for specific pathogenic viruses. The avian influenza viruses represent different pathogenicity and varying segmented genomes. The general inactivation mechanism (see section 3.5.2) of ammonia indicates that also NDV, belonging to the same *Paramyxovirinae* subfamily as BPIV 3, with only *approx*. 200 bp shorter genome, will be inactivated. Both feline coronavirus and avian bronchitis virus belongs to genus *Coronavirus*, thus have similar genome size. Regarding FCV, this virus has about 7.7kb compared to HEV and AHEV with approximately 7.2 kb and 6.6 kb, respectively. As recommendations were given based on the inactivation rate of bacteriophage MS2 with a 3.6 kb genome, which was shown to be inactivated about 5 times slower than FCV, these viruses are indicated to be inactivated also.

5.2 Food safety (Paper IV)

As a hazard in pork products, HEV has recently drawn attention through increasing cases of an autochthonous nature, including in Sweden (Widen *et al.*, 2011). In Paper IV swine liver, dry cured ham and cold smoked pork sausage were spiked with a spectrum of viruses as models for HEV, and subjected to the different treatments.

5.2.1 High pressure treatment

The results from the HPP treatments are shown in Table 7. Bacteriophage MS2 was the most stable microorganism, and did not show any significant reduction on swine liver tested up to 400 MPa. FCV wt was the most labile microorganism, and was inactivated by $\geq$5.0 $\log_{10}$ after 300 MPa. At 400 MPa, also FCV 2280 showed $\geq$4.2 $\log_{10}$ reduction, while MNV 1 was inactivated by 3.4$\pm$0.42(95%CI) $\log_{10}$.

In contrast, on dry cured ham at 400 MPa, MNV 1 was unaffected. Reductions on swine liver were significantly greater than on ham for the viruses at 300 and 400 MPa, and for ϕX174 to a lesser extent at 300 MPa.

Table 7. *Reductions (log_{10} $TCID_{50}$/PFU±95%CI) after 10 min high pressure treatments for murine norovirus 1, feline calicivirus 2280, feline calicivirus wt and bacteriophages MS2, øX174 and 28b on swine liver and dry cured ham at 12-13°C. Results are from two experiments with duplicate samples (Paper IV).*

Virus/phage	Liver 300 MPa	Liver 400 MPa	Ham 400 MPa	Ham 600 MPa
MNV 1	n.d.	3.4±0.42	0.11±0.81	2.9±0.88
FCV 2280	1.5±0.37	≥4.2±0.37[a]	0.70±0.38	4.1±0.80
FCV wt	≥5.0±0.92[a]	≥5.0±0.92[a]	1.5±0.66	4.4±0.64
MS2	-0.10±0.39	-0.12±0.39	-0.12±0.29	1.3±0.45
øX174	1.3±0.30	1.1±0.16	1.3±0.35	1.7±0.064
28b	0.29±0.19	0.52±0.13	0.5±0.28	1.7±0.30

n.d.=not done; [a]95%CI calculated from initial titre

5.2.2 Lactic acid treatment

Surface contamination at *e.g.* abattoirs could imply a HEV hazard. Lactic acid was tested as a surface treatment for spiked swine liver and dry cured ham for 10 min, with MNV 1 and FCV as models for HEV. MNV 1 showed initially higher reductions of 2.1±0.35(95%CI), for swine liver at 0.55 M lactic acid, and stabilized at lower pH values (Fig. 1). Bacteriophage øX174 was the most stable microorganism, and could be devised as a conservative indicator for MNV 1 at 0.55-1.1 M for swine liver, and at 0.55M for ham (Fig. 1). Bacteriophage MS2 and 28b were significantly more stable than MNV 1 only at 0.55M for swine liver. Feline calicivirus 2280 and FCV wt were the least stable microorganisms with 2.1±0.77(95%CI) and 1.9±0.78(95%CI) log_{10} reduction for swine liver, and 2.4±0.61(95%CI) and 3.1±0.29(95%CI) for ham, respectively, at 2.2 M lactic acid concentration (results not shown).

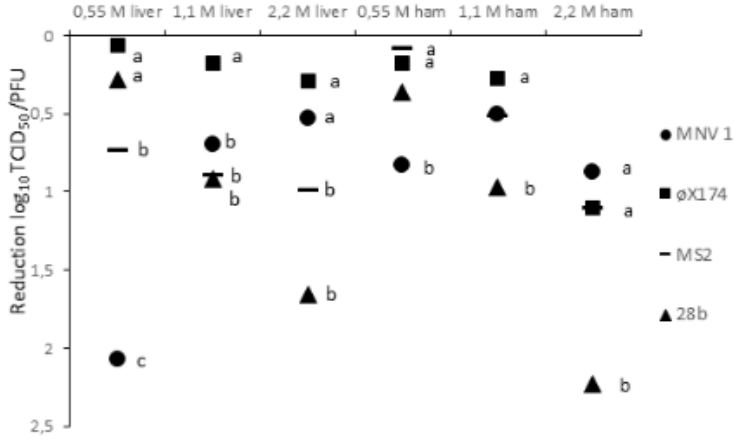

Figure 1. Reduction (log_{10} $TCID_{50}$ /PFU) in MNV 1 and bacteriophages øX174, MS2 and 28b, after lactic acid treatments for 10 min, on swine liver or dry cured ham. pH values at 0.55, 1.1 and 2.2 M final concentrations were 3.7±0.12, 3.4±0.10 and 3.1±0.20 for swine liver, and pH 3.6±0.11, 3.2±0.09 and 2.9±0.07 for ham. Values are means of three experiments, except for MNV 1 on swine liver, with six experiments. Different letters denote significant ($p<0.05$) differences between virus reductions for the respective food item and lactic acid concentration (Paper IV).

5.2.3 Intense light pulse treatment

Intense light pulse (ILP) treatment was performed in a laboratory Tecum-Mobile Decontamination Unit. Spiked samples of swine liver, dry cured ham and cold smoked sausage were subjected to different ILP-doses of 3-60 J cm^{-2}. Bacteriophage MS2 was the most stable microorganism, significantly more stable than one or both caliciviruses for swine liver and ham (Fig. 2). For sausage, one or both caliciviruses were significantly more labile than MS2 only at 12, 30 and 45 J cm^{-2}. Bacteriophages øX174 and 28b showed similar resistance as FCV 2280 (results not shown). Reductions on ham were significantly lower than on swine liver for øX174 at 3- 15 J cm^{-2} and for the other microorganisms at 15-60 J cm^{-2}.

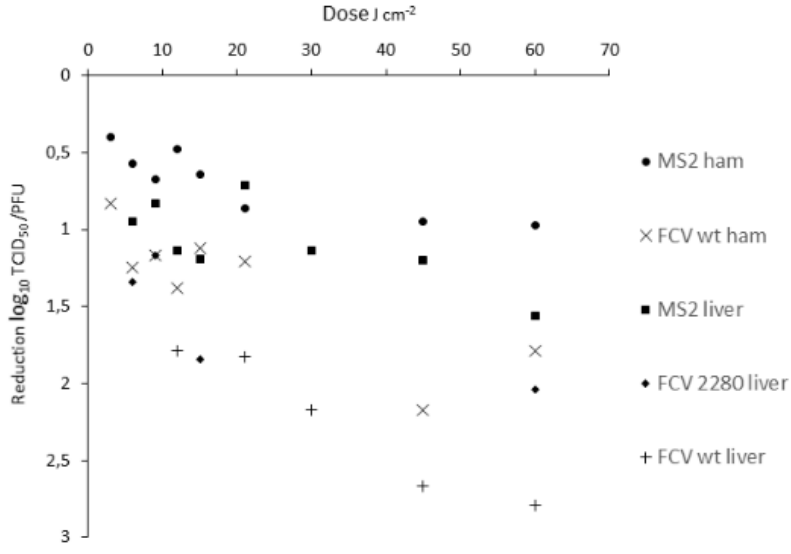

Figure 2. Reductions (log_{10} $TCID_{50}$ /PFU) in bacteriophage MS2, feline calicivirus (FCV) 2280 and FCV wild type (wt), after intense light pulse treatment with doses of 3, 6, 9, 12, 15, 21, 30, 45 and 60 J cm^{-2} on swine liver and dry cured ham. MS2 reductions were significantly ($p<0.05$) lower than for the caliciviruses on the respective food item (Paper IV).

5.2.4 Critical control points

High pressure processing is a non-thermal technology and has been shown to also inactivate internalized virus (Hirneisen & Kniel, 2013; Calci *et al.*, 2005). Reduction criteria for HEV is hard to estimate because actual virus levels in food and accurate dose-response relationship in humans are not known (Cook and van der Poel 2015). Based on our results, pressure and time influenced the virus reductions, and could be considered CCPs in treatment of swine liver and dry cured ham.

An important CCP is the temperature that together with pressure and time should be controlled continuously. Food texture has to be checked at these pressures. Other parameters influencing virus reduction could be pH, humidity and any chemicals such as salt content.

No reduction criteria are available for HEV regarding surface decontamination, although swine carcasses have been shown to contain up to 5.9 log_{10} 100 cm^{-2} according to PCR (Table 1). For LA treatment, the highest reduction in MNV 1 was achieved on swine liver at 0.55 M LA. For ILP

treatment, swine liver showed significantly higher reduction than ham for the caliciviruses at 15-60 J cm^{-2}. As HEV was found at high concentrations internally in swine liver (Table 1), this would present a hazard not reduced by surface treatment. However, ILP could be an alternative for decontamination of HAV or HuNoV in food handling.

5.2.5 Suitability of model viruses and bacteriophages

The approach was to use a spectrum of viruses. Murine norovirus showed higher stability to HPP in comparison to the feline caliciviruses. MNV 1 is also suggested as a more suitable norovirus surrogate (Kingsley *et al.*, 2007). In the HPP treatments on swine liver at 10 min, bacteriophages MS2 and 28b could be suggested as surrogates for norovirus and calicivirus at 300 MPa, including øX174 at 400 MPa. For dry cured ham at 600 MPa after 10 min, all bacteriophages were significantly more stable than the caliciviruses and MNV 1, and could be suggested as indicators for these kinds of viruses. However, MS2 and related bacteriophages showed higher inactivation at temperatures below 21 °C (Guan, Kniel *et al*. 2006; Guan, Joerger *et al*., 2007), in similarity to caliciviruses and noroviruses (Kingsley, 2013). This indicates that MS2 is a more conservative surrogate for these types of viruses. In contrast, the susceptibility of bacteriophage øX174 to HPP has been shown to be unaffected by temperatures of 4-40 °C (Guan, Kniel *et al*., 2006).
For lactic acid treatments, MNV is considered a more suitable norovirus surrogate due to its enteric nature (Wobus, Thackray *et al*., 2006). For swine liver, at 0.55 M LA addition, øX174, MS2 and 28b could be suggested as conservative indicators for MNV 1, the norovirus model for HEV, while at 1.1 M and 2.2 M only øX174 was an appropriate indicator. For dry ham at 0.55 M LA, both øX174 and MS2 were significantly more stable than MNV 1, and could be suggested as conservative indicators.

In the intense light pulse treatments on swine liver (Paper IV), MS2 was significantly more stable than one or both feline caliciviruses at 6-60 J cm^{-2}, indicating that it is a conservative surrogate for these types of viruses. On dry cured ham, only FCV wt was consistently more labile than MS2 and at 30 J cm^{-2} no significant difference of MS2 to the caliciviruses could be seen.

If the hazard is specified, preferably *relevant* model viruses of the same species should be chosen. However, as these viruses also are laboratory cultured, they may have changed their properties in regard to the pathogenic virus (EMEA, 1997). We saw significant differences between the two FCV strains in the HPP and the ILP studies, both for swine liver and ham, with FCV

wt being the most sensitive. In contrast, MNV 1 was shown as significantly more stable than the FCVs both for HPP and LA treatments.

Regarding UV treatment, FCV strain F9 is estimated as two- to three-fold more labile than MS2 (Hijnen *et al.*, 2006; Tree *et al.*, 2005), similarly to FCV wt in our ILP study. However, FCV 2280 was seen to be more stable. This strain also shows differences in capsid structure compared with strain F9 (Geissler *et al.*, 1997). Strains of *Vesivirus* have previously been shown to have varying susceptibility to UV treatment, but in comparison with MS2 this difference could be of minor importance (Husman *et al.*, 2004). Strain differences of FCV have also been seen against disinfectants (Di Martino *et al.*, 2010). This high-lights the fact that appropriate surrogate viruses could be a better choice.

5.3 Biosafety in an epizootic outbreak situation (Papers II and III)

In an epizootic outbreak situation of *e.g.* avian influenza or Newcastle disease virus, the waste materials, such as HW and manure, are classed as Category 2 materials and should thus at least be pressure-sterilised, as in any process requiring HACCP (EC, 2009). However, for biosafety reasons, it is often better to perform sanitation on-site, *e.g.* by liming, recommended at 40 kg m^{-3}, followed by storage for 4 days (Anon, 2009). In this respect, safer handling could be accomplished by NH_3 treatment, because it is easier to disperse in the materials. For the hatchery waste in Paper II, a sufficient reduction of 12 $\log_{10}$ was estimated based on the upper 99% confidence interval for the influenza viruses and the models for NDV and IBV at 14 °C after approximately 2 days at 0.5% (w/w) NH_3 addition (Table 8).

Another option is composting (Guan *et al.*, 2009), which can be performed in mesophilic (15-45 °C) or thermophilic (>45 °C) conditions. In Paper III, HPAIV H7N1 in mixtures of chicken manure and embryonated hen eggs was quickly reduced already at mesophilic temperatures of 35-45 °C (Table 7). The somewhat longer time for a 12 $\log_{10}$ reduction at 45 °C compared with 35 °C is due to the larger standard error of the inactivation rate constant in the regression, resulting in a larger confidence interval. At a thermophilic temperature of 55 °C, the time for a 12 $\log_{10}$ reduction was 30 min. Bacteriophages MS2 and ø6 were also tested as presumptive indicators, with estimated times for a 12 $\log_{10}$ reduction of 835 and 2 h, respectively (Table 8). In the laboratory composting reactors, both HPAIV H7N1 and ø6 were

inactivated below the detection limit within 24 h, while MS2 showed a 4-7 log_{10} PFU reduction after seven days.

Table 8. *Time to 12 log_{10} reduction (h) based on the upper 99% confidence intervals (99%CI), for ammonia (NH_3) addition of 0.5%(wt/wt), pH 9.5, NH_3 186 mmol kg^{-1} or 0.25%(wt/wt), pH 9.2, NH_3 74 mmol kg^{-1}, at 14°C and for composting at 35-55°C. Results from duplicate or triplicate experiments (Papers II and III)*

Virus/phage	Treatment	Material	D-value (99%CI)(h)	Time to 12 log_{10} reduction (h)
FCoV	NH_3 0.5%(wt/wt)	Hatchery waste	1.2(0.87-1.9)	23
BPIV 3	"	"	1.3(1.1-1.5)	18
FCV	"	"	2.0(1.6-2.7)	32
LPAIV	"	"	2.1(1.5-3.9)	47
HPAIV	"	"	3.0(2.3-4.3)	52
HPAIV	NH_3 0.25%(wt/wt)	"	4.1(3.0-6.2)	74
HPAIV	Heat 35°C	Compost mixture[a]	0.39(0.28-0.63)	7.6
HPAIV	Heat 45°C	"	0.12(0.07-0.82)	9.8
MS2	NH_3 0.5%(wt/wt)	Hatchery waste	7.7(5.9-12)	141
MS2	NH_3 0.25%(wt/wt)	"	14(12-17)	206
MS2	Heat 45°C	Compost mixture	62(55-70)	835
MS2	Heat 55°C	"	29(24-34)	403
ø6	Heat 35°C	"	0.17(0.10-0.44)	5.3
ø6	Heat 45°C	"	0.11(0.09-0.17)	2.0

[a]Chicken manure, straw and embryonated hen eggs.

5.3.1 Critical control points and critical limits

Ammonia treatment could be used for inactivation of influenza viruses and models for epizootic viruses. The CCPs would be pH, temperature and time. As an example, for HPAIV H7N1, with NH_3 addition to 0.5%(wt/wt), the critical time limit would be >52 h at a temperature of >14 °C and a pH of >9.5. These should be measured preferably continuously.

For the composting, the surface temperature is considered a CCP. Based on the validation by HPAIV H7N1, the critical limits would be a surface temperature of >35 °C for a period of >7.6 h. The temperature should be measured continuously.

5.3.2 Suitability of model viruses and bacteriophages

For suitability of model viruses for ammonia treatment, see section 5.1.4.

In the composting study, in the handling of epizootic waste, *relevant* or *specific* model viruses are used, thus the strain HPAIV A/turkey/Italy/1387/00(H7N1) was chosen, as it had shown heat stability (Terregino *et al.*, 2009), with bacteriophage ø6 with similar structure, showing similar inactivation rate. In the composting study (Paper III), MS2 was found to be very heat stable, and too stable as an indicator for HPAIV H7N1 inactivation during these conditions. The calculated time for a 12 $\log_{10}$ reduction (99%CI) would be 14.4 and 16.8 days in the two compost mixtures even at 55 °C. This confirms claims that these types of bacteriophages show stability at higher temperatures, >50 °C (Bertrand *et al.*, 2012).

The laboratory composting study also showed fast inactivation of HPAIV H7N1 within 24 h with a >3 $\log_{10}$ reduction, while MS2 showed a 4 and 7 $\log_{10}$ reduction after 7 days in the two mixtures, respectively. In contrast, bacteriophage ø6 showed similar resistance as H7N1, with 2-5.4 h (99%CI) to a 12 $\log_{10}$ reduction at mesophilic temperatures of 35-45 °C. Due to its non-pathogenicity, ease of handling and low cost, bacteriophage ø6 is a good alternative for validating H7N1 inactivation in these conditions. However, MS2 could be an alternative indicator for more stable enteric viruses.

5.4 Managing the risk

All studies presented in this thesis investigated virus reduction treatments in laboratory-scale studies controlling the factors considered important for virus inactivation. By examining the effect on viral reduction as a function of these factors, the intention was to formulate recommendations on the levels of these parameters that should be kept in the processes over a certain time to reach sufficient viral reductions.

We have used a laboratory scale approach, where we could assess the CCPs that could be measured. This approach has also the advantage that the inactivation of the identified virus hazards could be directly compared with that of a model virus. If bacteriophages are used as models, they can subsequently be used as process indicators in the actual process. For validation of food safety processes, consideration must be given to ensuring that the validation process reflects the production-scale process (Busta *et al.*, 2003). For biowaste, considerations for upscaling also have to be taken, as the intrinsic properties of the materials can differ substantially (Elving *et al.*, 2014).

5.4.1 Considerations for upscaling

For the identified CCPs and critical limits for the treatments under normal circumstances see section 5.1.3.

Thermal treatment

In Paper I, the SVDV reduction in biogas substrates, based on the lower 95% confidence interval at 70 °C for 30 min, was >5 $\log_{10}$ reduction, a criterion for a less thermally resistant virus (EFSA, 2005). Thus, most of the viral hazards except parvovirus and circovirus would be inactivated (Table 1).

Two controlled time points allowed only estimation of D-values. At a temperature of 55 °C, an estimated D-value of 28 min for SVDV indicates that bacteriophage ϕX174, which showed a D-value of 1.5 h (Elving *et al.*, 2014), could be a useful indicator. As naturally occurring virus may have a lower inactivation rate (4- to 10-fold lower) than added virus (Lund *et al.*, 1996), øX174 as a somatic coliphage has the advantage of being present in biowaste in high amounts. Bacteriophage øX174 was also seen as highly persistent in various matrices and temperatures (Bertrand *et al.*, 2012). Bacteriophage MS2 in the composting experiments (Paper III) had a D-value of about 24 h (Paper III), and would be a more conservative indicator.

Ammonia treatment

Ammonia treatment gave D-values of 5-13 hours and 3-7 hours (upper 95%CI) at 0.25% and 0.5% (wt/wt) ammonia addition, respectively, at 5 °C for FCoV, LPAIV H5N3, FCV and HPAIV H7N1, the latter being the most resistant. However, for BPIV-3 at 5 °C, D-values (upper 95%CI) were 20-12 h at 0.25-0.75%(wt/wt), indicating longer treatment times for BPIV-3 at low temperatures.

The large-scale trial at a hatchery regarding ammonia treatment of HW 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. Thus this would be within the critical limits for the recommended target of 3 $\log_{10}$ reduction (EC, 2011), *i.e.* addition of 0.25% (w/w) NH_3 (10 L 28% NH_3 [aq] m^{-3} material) followed by storage for 2 days at 14 °C (Table 6) (critical limits: temperature >14°C, pH >9.2, time >2 days). 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. Larger pieces of material such as embryos and culled chicks that can be present in the HW should not constitute a problem, since NH_3 diffuses easily through biological material (Warren, 1962). However, longer treatment times might be needed if the material is not pre-minced.

For verification of HACCP procedures of these processes, available options include the insertion of test containers with *e.g.* parvovirus, provided by test laboratories (Amlinger & Blytt, 2013).

Food treatments

In Paper IV, the identified hazard was swine HEV. Swine liver and swine carcasses has been shown to contain approximately 6 $\log_{10} g^{-1}$/100 cm^{-2} as estimated by PCR, respectively (Table 1). Reduction factors with 95%CI were calculated at the respective time/pressure combinations, and pressure and time could be suggested as critical control points based on the reduction of the models (section 5.2.4).

However, this approach only allows defined production steps to be validated. Instead, D-values could be more easily used in the HACCP plans in the production facilities in the food chain (Zuber *et al.*, 2013). Regarding food safety processes, D-values have been used *e.g*. to assess virus inactivation by heat (time to 1 $\log_{10}$ reduction) (Swayne & Beck, 2004) or irradiation (dose to 1 $\log_{10}$ reduction) (Brahmakshatriya *et al.*, 2009) in poultry products. However, in the ILP experiments in Paper IV, a first-order regression was seen only at 3-12 J cm^{-2} for some virus/food combinations and for HPP, after onset of reduction to some extent.

For upscaling of HPP treatment, larger pieces of food could be used, since HPP affects the whole food item. The CCPs temperature, pressure and time must be monitored according to critical limits implemented in the HACCP plan. If the production facility allows, bacteriophages could be used to validate the process.

5.4.2 Epizootic outbreak situation

For the identified CCPs and critical limits for the treatments during an outbreak situation see paragraph 5.3.1.

In virus validations (EMEA, 1997), separate inactivation steps are added if they are due to different inactivation mechanisms, so as to reach *e.g.* a 12 $\log_{10}$ reduction in the final product (Aranha, 2001). This was also the strategy for assessing safety in outbreak situations. Thus, in Papers II and III, in controlled conditions, D-values with 99% confidence intervals were calculated and treatment times for a 12 $\log_{10}$ reduction were estimated from the upper confidence interval limit. Safety margins must be set due to *e.g.* non-homogeneity of the biowaste and because the actual virus content is often not known.

For ammonia treatment of hatchery waste (Paper II), an initial estimate of the volume of the infected materials and proper mixing of the ammonia is crucial. Since NH_3 is the active component, it is important that it is not lost as gaseous emissions and therefore treatment must take place in a closed container, preferably on-site. In these circumstances, special equipment that is easy to decontaminate should preferably be used for the CCPs temperature and pH measurement.

Similar biosafety considerations apply for the composting procedure (Paper III), with minimal turning during the first stage of composting, as this might result in spreading of the pathogens. Arrangements to ensure the temperature have to be taken. Surface temperature as a CCP should be measured continuously as to monitor the critical limit.

6 Conclusions

The inactivation of *relevant* and *specific* model viruses was assessed at controlled conditions, and compared with that of conservative indicator viruses. Based on statistical evaluations, treatment recommendations for biowaste and food regarding physical and chemical inactivation methods were formulated:

- 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. Composting was found to be an effective measure for disinfecting poultry manure and egg litter contaminated with highly pathogenic avian influenza. Based on the D-values with their 99% confidence intervals, treatment recommendations for outbreak situations regarding these viruses could be given, in order to minimise the risk of viral pathogenic transmission via contaminated litter. Bacteriophage ø6 could be a useful model in influenza virus composting.
- The bacteriophage MS2 proved to be an excellent indicator for ssRNA virus inactivation regarding ammonia in hatchery waste. Based on the D-values with their 95% confidence interval, 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, was assessed as a control measure for inactivation of swine vesicular disease virus. However, the thermostable porcine parvovirus proved very heat resistant. If this virus were to be used as a 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 such as øX174 or PRD 1 could be used as indicators, if validated against relevant viruses.

- High pressure processing at 400 MPa on swine liver and at 600 MPa on dry cured ham showed highest inactivations of the norovirus and calicivirus models for hepatitis E virus, with bacteriophages MS2, 28B and øX174 as conservative process indicators. Based on the reduction factors with their 95% confidence intervals, critical limits for pressure level and time at a defined temperature could be set for their inactivation.
- Surface decontamination by lactic acid at 0.55M on swine liver was the most effective regarding the norovirus model for hepatitis E virus, with bacteriophages MS2, 28B and øX174 as conservative indicators. For the calicivirus models, 2.2 M lactic acid was the most effective, with øX174 as a conservative indicator for swine liver, and øX174 and MS2 for dry cured ham.
- Intense light pulse treatment was most effective on swine liver, and reduced the surface contamination of the calicivirus models for hepatitis E virus at 6-60 J cm^{-2}, with bacteriophage MS2 as a conservative indicator.
- However, hepatitis E virus has been found at high concentrations internally in swine liver and this would present a substantial hazard not reduced by surface treatments.

7 Future perspectives

Regarding ammonia inactivation, other types of viruses could be tested during treatment of hatchery waste, because there seem to be other inactivation mechanisms involved for double-stranded viruses and DNA-viruses.

To evaluate inactivation of viruses that are difficult to culture, such as HEV, PCR-based methods combined with viability dyes such as propidium monoazide have been used, to discriminate between active and inactive virus. These methods are based on the fact that the capsid is affected by such inactivation methods like heat. This could also be an alternative for HEV regarding these inactivation methods. Other methods are based on the receptor-binding properties of intact virus particles, and capture before PCR analysis. These methods could also be evaluated regarding HEV for appropriate treatments.

8 Populärvetenskaplig sammanställning

Animaliska biprodukter innehåller värdefulla växtnäringsämnen, som kan återvinnas genom gödsling av åkermark. Dessa material kan innehålla smittämnen, t.ex. virus, som kan innebära en risk för lantbruksdjur och vilda djur, såväl som för människor vidare i livsmedelskedjan. För livsmedel kan virussmitta introduceras i alla produktionssteg, från slakt till paketering och distribution. För att minska riskerna, kan olika behandlingar användas, fysikaliska såsom värme och högt tryck, eller kemiska t.ex. ammoniakbehandling eller organiska syror. Eftersom många viktiga livsmedelsburna virus inte går att odla, är den enda möjligheten för att utvärdera effekten av virusinaktiveringsmetoder att använda modeller för de sjukdomsframkallande virusen. Som virusmodeller kan även bakteriofager, virus som infekterar bakterier, användas, eftersom de har liknande struktur.

Virus som smittar från djur till människa är t.ex. aviärt influensavirus och hepatit E virus (HEV) från gris. Som modeller för HEV användes felint calicivirus och musnorovirus, och bakteriofagerna MS2, øX174 och 28b, i behandlingar av grisprodukter med högt tryck, mjölksyra och intensiva ljuspulser. Som modeller för högpatogent aviärt influensavirus användes bakteriofagerna MS2 och ø6 i ammoniakbehandling och kompostering av animaliska biprodukter.

I laboratorieförsök kontrollerades de faktorer som ansågs vara de viktigaste för virusinaktiveringen, såsom trycknivå, pH, ljuspulser och temperatur, och reduktionen av sjukdomsframkallande virus och deras modeller bestämdes som en funktion av dessa faktorer. Rekommendationer kunde ges om vilka kontinuerligt mätbara processförhållanden som bör hållas under en viss tid för att nå tillräcklig reduktion av virus, baserade på statistiska bedömningar av laboratorieresultat, både för normala förhållanden och i en epizootisk utbrottssituation. Bakteriofagerna undersöktes för användning som potentiella indikatorer för kontroll eller validering av fullskaliga processer.

Resultaten gav behandlingsrekommendationer för att säkerställa en tillräcklig virusreduktion enligt gällande EU-krav för animala biprodukter kategori 3 (2011/142/EG) för ammoniak och värmebehandling. För en utbrottssituation kunde rekommendationer ges för hantering av högpatogent aviärt influensavirus, baserade på att pH och temperatur ska upprätthållas under en viss tid för hanteringen. I högtrycksbehandling av grisprodukter kunde tryck och tid definieras som kritiska kontrollpunkter för felint calicivirus och musnorovirus, modeller för hepatit E virus.

I större skala kunde MS2 och ø6 användas för verifiering av ammoniakbehandling av kläckeriavfall och i laboratoriekompostering. I behandlingar av grisprodukter, visade det sig att bakteriofagerna hade potential som stabila modeller för noro och calicivirus i större produktionsskala, med MS2 som den mest stabila indikatorn i högtrycksbehandlingar och behandlingar med intensiva ljuspulser, och øX174 i mjölksyrabehandlingar.

References

Adams, M.H. (1959). *Bacteriophages*: Inter-science Publishers, Inc, New York.

Adcock, N.J., Rice, E.W., Sivaganesan, M., Brown, J.D., Stallknecht, D.E. & Swayne, D.E. (2009). The use of bacteriophages of the family Cystoviridae as surrogates for H5N1 highly pathogenic avian influenza viruses in persistence and inactivation studies. *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering*, 44(13), pp. 1362-66.

Albihn, A. & Vinneras, B. Biosecurity and arable use of manure and biowaste - treatment alternatives. In: *Proceedings of 12th International Conference of the Ramiran Network, Aarhus, Denmark, 11-23 September 2006*.2007, pp. 232-239.

Albihn, A., Nyberg, K., Ottoson, J. & Vinnerås, B. (2012). Sanitation Treatment Reduces the Biosecurity Risk when Recycling Manure and Biowaste. In: Jacobsson, C. (Ed.) *Ecosystem Health and Sustainable Agriculture Book 1*. pp. 136-141.

Alexander, D.J. & Senne, D.A. (2008). Newcastle Disease. In: Saif, Y.M. (ed. *Diseases of Poultry*. 12. ed. Ames: Blackwell Publishing, pp. 75-100.

Amlinger, F. & Blytt, L.D. (2013). *Good Practice Guide-How to comply with the EU Animal By-Products Regulations at Composting and Anaerobic Digestion Plants*. Bochum, Germany: European Compost Network

Anon (1995). *ISO 10705-1, Water quality - Detection and enumeration of bacteriophages - Part 1: Enumeration of F-specific RNA bacteriophages.* Geneva: International Standardisation Organisation.

Anon (2000). *ISO 10705-2 Water quality - Detection and enumeration of bacteriophages - Part 2: Enumeration of somatic coliphages*. Geneva: International Standardisation Organisation.

Anon (ed.) (2005). *Virus Taxonomy Eight Report of the International Committee on Taxonomy of Viruses*. Virus Taxonomy. San Diego: Elsevier Academic Press.

Anon (2009). *Epizootihandboken*: Swedish Board of Agriculture. Available from: http://www.jordbruksverket.se/amnesomraden/djur/djurhalsopersonal/epizootihandboken.106.160b021b1235b6bb86180003962.html.

Aranha, H. (2001). Viral Clearance Strategies for Biopharmaceutical Safety, part II: A Multifaceted Approach to Process Validation. *BioPharm,* 14(5), pp. 43-54,90.

Baechlein, C., Seehusen, F., Nathues, H., Beilage, E., Baumgartner, W. & Grummer, B. (2013). Molecular detection of hepatitis E virus in German domestic pigs. *Berl Munch Tierarztl Wochenschr,* 126(1-2), pp. 25-31.

Barnaud, E., Rogee, S., Garry, P., Rose, N. & Pavio, N. (2012). Thermal Inactivation of Infectious Hepatitis E Virus in Experimentally Contaminated Food. *Applied and Environmental Microbiology,* 78(15), pp. 5153-59.

Bartolo, I. d., Diez-Valcarce, M., Vasickova, P., Kralik, P., Hernandez, M., Angeloni, G., et al. (2012). Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. *Emerging Infectious Diseases,* 18(8), 1282-89.

Beato M. S., Mancin M., Bertoli E., Buratin A., Terregino C. & Capua I. (2015). Infectivity of H7 LP and HP influenza viruses at different temperatures and pH and persistence of H7 HP virus in poultry meat at refrigeration temperature. *Virology*, 433(2), 522-7.

Belak, S., Karlsson, O.E., Blomstrom, A.L., Berg, M. & Granberg, F. (2013). New viruses in veterinary medicine, detected by metagenomic approaches. *Veterinary Microbiology,* 165(1-2), pp. 95-101.

Belliot, G., Loutreul, J., Estienney, M., Cazeaux, C., Nicorescu, I., Aho, S., Gervais, P., Orange, N., Pothier, P. & Morin, T. (2013). Potential of Pulsed Light to Inactivate Bacteriophage MS2 in Simple Liquid Medium and on Complex Foodstuffs. *Food and Environmental Virology*. Mar 23. [Epub ahead of print]

Berry, M., Gamieldien, J. & Fielding, B.C. (2015). Identification of new respiratory viruses in the new millennium. *Viruses*, 7(3), pp. 996-1019.

Berto, A., Martelli, F., Grierson, S., & Banks, M. (2012). Hepatitis E Virus in Pork Food Chain, United Kingdom, 2009-2010. *Emerging Infectious Diseases*, 18(8), pp. 1358-60.

Bertrand, I., Schijven, J.F., Sanchez, G., Wyn-Jones, P., Ottoson, J., Morin, T., Muscillo, M., Verani, M., Nasser, A., de Roda Husman, A.M., Myrmel, M., Sellwood, J., Cook, N. & Gantzer, C. (2012). The impact of temperature on the inactivation of enteric viruses in food and water: a review. *Journal of Applied Microbiology,* 112(6), pp. 1059-74.

Black, E.P., Cascarino, J., Guan, D., Kniel, K.E., Hicks, D.T., Pivarnik, L.F. & Hoover, D.G. (2010). Coliphage as pressure surrogates for enteric viruses in foods. *Innovative Food Science & Emerging Technologies*, 11(2), pp. 239-244.

Blumel, J., Schmidt, I., Willkommen, H. & Lower, J. (2002). Inactivation of parvovirus B19 during pasteurization of human serum albumin. *Transfusion*, 42(8), pp. 1011-8.

Boschetti, N., Wyss, K., Mischler, A., Hostettler, T. & Kempf, C. (2003). Stability of minute virus of mice against temperature and sodium hydroxide. *Biologicals.* 31(3), pp. 181-5.

Bouwknegt M., Rutjes S. A., Reusken C. B., Stockhofe-Zurwieden N., Frankena K., de Jong M. C., de Roda Husman A. M. & Poel W. H. (2009). The course of hepatitis E virus infection in pigs after contact-infection and intravenous inoculation. *BMC Veterinary Research*. doi: 10.1186/1746-6148-5-7

Brahmakshatriya, V., Lupiani, B., Brinlee, J.L., Cepeda, M., Pillai, S.D. & Reddy, S.M. (2009). Preliminary study for evaluation of avian influenza virus inactivation in contaminated poultry products using electron beam irradiation. *Avian Pathology*, 38(3), pp. 245-50.

Brankston, G., Gitterman, L., Hirji, Z., Lemieux, C., Gardam, M. (2007). Transmission of influenza A in human beings. *Lancet Infectious Diseases*, 7(4), pp. 257-265.

Brassard, J., Gagne, M. J., Genereux, M. & Cote, C. (2012). Detection of human food-borne and zoonotic viruses on irrigated, field-grown strawberries. *Applied and Environmental Microbiology*, 78(10), pp. 3763-6.

Breum, S.O., Hjulsager, C.K., de Deus, N., Segales, J. & Larsen, L.E. (2010). Hepatitis E virus is highly prevalent in the Danish pig population. *Veterinary Microbiology*, 146(1-2), pp. 144-9.

Brewer, L.A., Lwamba, H.C.M., Murtaugh, M.P., Palmenberg, A.C., Brown, C. & Njenga, M.K. (2001). Porcine encephalomyocarditis virus persists in pig myocardium and infects human myocardial cells. *Journal of Virology*, 75(23), pp. 11621-11629.

Brown Jr, T. T. (1981). Laboratory evaluation of selected disinfectants as virucidal agents against porcine parvovirus, pseudorabies virus, and transmissible gastroenteritis virus. *American Journal of Veterinary Research*. 42(6), pp. 1033-36.

Burge, W.D., Cramer, W.N. & Kawata, K. (1983). Effect of heat on virus inactivation by ammonia. *Applied and Environmental Microbiology*, 46(2), pp. 446-51.

Busta, F.F., Suslow, T.V., Parish, M.E., Beuchat, L.R., Farber, J.N., Garrett, E.H. & Harris, L.J. (2003). The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, 2(1 SUPPL.), pp. 179-185.

Böhm, R. Problems connected with the EU-animal by-product regulations in the field of environmental and animal hygiene. In: M. Pilar Bernal, R.M., Rafael Clemente, Concepcion Paredes (ed. *Proceedings of Ramiran 2004: Sustainable Organic Waste Management for Environmental Protection and Food Safety*, Murcia, Spain2005: Tipografia San Francisco, S.A. Murcia, Spain, pp. 49-52.

Bötner, A. & Belsham, G. J. (2012). Virus survival in slurry: analysis of the stability of foot-and-mouth disease, classical swine fever, bovine viral diarrhoea and swine influenza viruses. *Veterinary Microbiology*, 157(1-2), pp. 41-9.

CAC/RCP 1-1969 - Annex Hazard Analysis and Critical Control Point (HACCP) System and Guidelines for its Application (2003).

Calci, K.R., Meade, G.K., Tezloff, R.C. & Kingsley, D.H. (2005). High-pressure inactivation of hepatitis A virus within oysters. *Applied and Environmental Microbiology*, 71(1), pp. 339-343.

Cao, D. & Meng, X. J. (2012). Molecular biology and replication of hepatitis E virus. *Emerging Microbes and Infection*, 1(8), s. e17.

Cappucci, D.T., Jr., Johnson, D.C., Brugh, M., Smith, T.M., Jackson, C.F., Pearson, J.E. & Senne, D.A. (1985). Isolation of avian influenza virus (subtype H5N2) from chicken eggs during a natural outbreak. *Avian Diseases*, 29(4), pp. 1195-200.

Capua, I., Scacchia, M., Toscani, T., Caporale V. (1993). Unexpected isolation of virulent Newcastle disease virus from commercial embryonated fowls' eggs. *Zentralbl Veterinarmed B.*, 40 Dec(9-10), pp. 609-612.

Carrington, E.G. (2001). *Evaluation of sludge treatments for pathogen reduction-Final report.* Luxembourg: European Commission.

Cavanagh, D. & Gelb Jr, J. (2008). Infectious Bronchitis. In: Saif, Y.M. (ed. *Diseases of Poultry*. 12. ed. Ames: Blackwell Publishing, pp. 117-135.

CDC (June 11). (2015a) Highly Pathogenic Asian Avian Influenza A (H5N1) in People. http://www.cdc.gov/flu/avianflu/h5n1-people.htm.

CDC (June 11). (2015b) *Avian Influenza Current Situation*. http://www.cdc.gov/flu/avianflu/avian-flu-summary.htm.

Chan, P.K. (2002). Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. *Clinical Infectious Diseases*, 34(Suppl 2), pp. 58-64.

Chandran, A., Pradhan, S.K. & Heinonen-Tanski, H. (2009). Survival of enteric bacteria and coliphage MS2 in pure human urine. *Journal of Applied Microbiology*, 107(5), pp. 1651-7.

Chen, J.P. & Wang, C.H. (2002). Clinical epidemiologic and experimental evidence for the transmission of Newcastle disease virus through eggs. *Avian Diseases*, 46(2), pp. 461-5.

Chen H., Hoover D. G. & Kingsley D. H. (2005). Temperature and treatment time influence high hydrostatic pressure inactivation of feline calicivirus, a norovirus surrogate. *Journal of Food protection*, 68(11), pp. 2389-94.

Choi, H. J., Song, J. H., Bhatt, L. R., & Baek, S. H. (2010). Anti-human rhinovirus activity of gallic acid possessing antioxidant capacity. *Phytotherapy Research*, 24(9), 1292-96.

Colson, P., Borentain, P., Queyriaux, B., Kaba, M., Moal, V., Gallian, P., Heyries, L., Raoult, D. & Gerolami, R. (2010). Pig Liver Sausage as a Source of Hepatitis E Virus Transmission to Humans. *Journal of Infectious Diseases*, 202(6), pp. 825-834.

Cook, N., & van der Poel, W. H. (2015). Survival and Elimination of Hepatitis E Virus: A Review. *Food and Environmental Virology*, 7(3), 189-194.

CAC/GL 69 - 2008. *Guidelines for the Validation of Food Safety Control Measures.* Codex Alimentarius Commission.

Cramer, W.N., Burge, W.D. & Kawata, K. (1983). Kinetics of virus inactivation by ammonia. *Applied and Environmental Microbiology*, 45(3), pp. 760-5.

De Benedictis, P., Beato, M.S. & Capua, I. (2007). Inactivation of avian influenza viruses by chemical agents and physical conditions: a review. *Zoonoses Public Health*, 54(2), pp. 51-68.

Decrey, L., Kazama, S., Udert, K. M. & Kohn, T. (2015). Ammonia as an in situ sanitizer: inactivation kinetics and mechanisms of the ssRNA virus MS2 by NH_3. *Environmental Science and Technology*, 49(2), pp. 1060-7

Decrey, L. (2015). *Virus inactivation mechanisms in human urine and fecal sludge.* Diss. Lausanne: École Polytechnique Fédérale de Lausanne.

Derbyshire, J.B., Monteith, H.D. & Shannon, E.E. (1986). Virological studies on an anaerobic digestion system for liquid pig manure. Agricultural Wastes 18(4), 309-312.

Derbyshire, J.B. (1989). Porcine Enterovirus (Polioencephalomyelitis). In: Marian C, Horzinek (Ed.). Virus Infections of Vertebrates. Amsterdam. Elsevier. Vol 2 p. 225

DiCaprio, E., Culbertson, D., & Li, J. (2015). Evidence of the Internalization of Animal Caliciviruses via the Roots of Growing Strawberry Plants and Dissemination to the Fruit. *Applied and Environmental Microbiology*, 81(8), 2727-34.

Di Martino, B., Ceci, C., Di Profio, F. & Marsilio, F. (2010). In vitro inactivation of feline calicivirus (FCV) by chemical disinfectants: resistance variation among field strains. *Archives of Virology*, 155(12), pp. 2047-51.

Dimopoullos, G.T., Fellowes, O.N., Callis, J.J., Poppensiek, G.C., Edward, A.G. & Graves, J.H. (1959). Thermal inactivation and antigenicity studies of heated tissue suspensions containing foot-and-mouth disease virus. *American Journal of Veterinary Research*, 20, pp. 510-21.

EC (2009). Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation)

EC (2011). Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive.

EFSA (2005). Opinion of the Scientific Panel on Biological Hazards of the European Food Safety Authority on the safety vis-a-vis biological risks of biogas and compost treatment standards of animal by-products (ABP). *The EFSA Journal*, 264, pp. 1-21.

EFSA (2011). Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *The EFSA Journal*, 9(7):2190 [96 pp.].

Ellis, J.R. & McCalla, T.M. (1978). Fate of pathogens in soils receiving animal wastes - a review. *Transactions of the ASAE*, 21(2), 309-13.

Elving, J., Vinneras, B., Albihn, A. & Ottoson, J.R. (2014). Thermal treatment for pathogen inactivation as a risk mitigation strategy for safe recycling of organic waste in agriculture. *Journal of Environmental Science and Health, Part B.*, 49(9), pp. 679-89.

EMEA (1996). Note for Guidance on Virus Validation Studies: the Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses. *CPMP/BWP/268/95*. London: The European Agency for the Evaluation of Medicinal Products.

EMEA (1997). Quality of biotechnological products: viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. *ICH Topic Q 5 A (R1).* London: European Medicines Agency.

Emerson, K., Russo, R., Lund, R. & Thurston, R. (1975). Aqueous Ammonia Equilibrium Calculations: Effects of PH and Temperature. *Journal of the Fisheries Research Board of Canada*, 32, pp. 2379-83.

Emerson, S.U., Arankalle, V.A. & Purcell, R.H. (2005). Thermal stability of hepatitis E virus. *Journal of Infectious Diseases*, 192(5), pp. 930-3.

Emmoth, E., Dergel, I., McNeilly, F., Allan, G.M., Albihn, A., Klingeborn, B. Heat inactivation of Porcine circovirus type 2. In: M. Pilar Bernal, R.M., Rafael Clemente, Concepcion Paredes (ed. *Proceedings of Ramiran 2004: Sustainable Organic Waste Management for Environmental Protection and Food Safety*, Murcia, Spain2005: Tipografia San Francisco, S.A. Murcia, Spain, pp. 207-210

FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Oranization].2008. *Viruses in Food: scientific advice to support risk management activities.* Meeting Report. Microbiological Risk Assessment Series No. 13. Rome. 58pp.

Feagins, A.R., Opriessnig, T., Guenette, D.K., Halbur, P.G. & Meng, X.J. (2008). Inactivation of infectious hepatitis E virus present in commercial pig livers sold in local grocery stores in the United States. *International Journal of Food Microbiology*, 123(1/2), pp. 32-37.

Field, H. E., Mackenzie, J. S. & Daszak, P.. (2007). Henipaviruses: emerging paramyxoviruses associated with fruit bats. *Current Topics in Microbiology and Immunology*. 315, pp. 133-59.

Fong, T.T. & Lipp, E.K. (2005). Enteric viruses of humans and animals in aquatic environments: health risks, detection, and potential water quality assessment tools. *Microbiology and Molecular Biology Reviews*, 69(2), pp. 357-71.

Geissler, K., Schneider, K., Platzer, G., Truyen, B., Kaaden, O.R. & Truyen, U. (1997). Genetic and antigenic heterogeneity among feline calicivirus isolates from distinct disease manifestations. *Virus Research*, 48(2), pp. 193-206.

Gentry-Shields, J., Myers, K., Pisanic, N., Heaney, C. & Stewart, J. (2015). Hepatitis E virus and coliphages in waters proximal to swine concentrated animal feeding operations. *Science of the Total Environment*. 505, pp. 487-93.

Gomez-Lopez, V. M., Devlieghere, F., Bonduelle, V. & Debevere, J. (2005). Factors affecting the inactivation of micro-organisms by intense light pulses. *Journal of Applied Microbiology*, 99(3), pp. 460-70.

Gomez-Lopez, V. M., Ragaert, P., Debevere, J. & Devlieghere, F. (2007). Pulsed light for food decontamination: a review. *Trends in Food Science & Technology*, 18(9), pp. 464-73.

Graiver D. A., Topliff C. L., Kelling C. L. & Bartelt-Hunt S. L. (2009). Survival of the avian influenza virus (H6N2) after land disposal. *Environmental Science & Technology,* 43(11), pp. 4063-7.

Guan, D., Kniel, K., Calci, K. R., Hicks, D. T., Pivarnik, L. F., & Hoover, D. G. (2006). Response of four types of coliphages to high hydrostatic pressure. *Food Microbiology,* 23(6), 546-551.

Guan, D., Joerger, R. D., Kniel, K. E., Calci, K. R., Hicks, D. T., Pivarnik, L. F., et al. (2007). Effect of high hydrostatic pressure on four genotypes of F-specific RNA bacteriophages. *Journal of Applied Microbiology,* 102(1), 51-56.

Guan, J., Chan, M., Grenier, C., Wilkie, D.C., Brooks, B.W. & Spencer, J.L. (2009). Survival of avian influenza and Newcastle disease viruses in compost and at ambient temperatures based on virus isolation and real-time reverse transcriptase PCR. *Avian Diseases,* 53(1), pp. 26-33.

Guo, H., Zhou, E.M., Sun, Z.F. & Meng, X.J. (2007). Egg whites from eggs of chickens infected experimentally with avian hepatitis E virus contain infectious virus, but evidence of complete vertical transmission is lacking. *Journal of General Virology,* 88(Pt 5), pp. 1532-7.

Haas, B., Ahl, R., Bohm, R. & Strauch, D. (1995). Inactivation of viruses in liquid manure. *Revue Scientifique et Technique - Office International des Epizooties,* 14(2), pp. 435-445.

Hagiwara, K., Iwabu, Y., Kanai, Y., Miyasho, T., Daidoji, T., Yunoki, M., Tsujikawa, M., Ohkubo, Y., Yasue, H. & Ikuta, K. (2007). Distribution and propagation of hepatitis E virus in experimentally infected swine. *The Open Veterinary Science Journal,* 1, pp. 1-6(6).

Hansen, K.H., Angelidaki, I. & Ahring, B.K. (1998). Anaerobic digestion of swine manure: Inhibition by ammonia. *Water Research,* 32(1), pp. 5-12.

Havelaar, A.H., Butler, M., Farrah, S.R., Jofre, J., Marques, E., Ketratanakul, A., Martins, M.T., Ohgaki, S., Sobsey, M.D. & Zaiss, U. (1991). Bacteriophages as model viruses in water quality control. *Water Research,* 25(5), pp. 529-545.

Herbert, T. P., Brierley, I. & Brown, T. D. (1997). Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation. *Journal of General Virology*, 78(Pt5), pp. 1033-40.

Herniman, K.A., Medhurst, P.M., Wilson, J.N. & Sellers, R.F. (1973). The action of heat, chemicals and disinfectants on swine vesicular disease virus. *Veterinary Record,* 93(24), pp. 620-4.

Hijnen, W.A.M., Beerendonk, E.F. & Medema, G.J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. *Water Research (Oxford),* 40(1), pp. 3-22.

Hirneisen, K.A., Hoover, D.G., Hicks, D.T., Pivarnik, L.F. & Kniel, K.E. (2012). Pressure inactivation of enteric viruses in a seafood salad-like product. *Journal of Aquatic Food Product Technology,* 21(5), pp. 455-467.

Hirneisen, K.A. & Kniel, K.E. (2013). Inactivation of internalized and surface contaminated enteric viruses in green onions. *International Journal of Food Microbiology,* 166(2), pp. 201-6.

Holmqvist, A. & Stenström, T. A. (2002). Survival of Ascaris suum ova, indicator bacteria and Salmonella Typhimurium phage 28b in mesophilic composting of household waste. *EcoSanRes 2002*.

Holmqvist, A., Emmoth, E., Albihn, A. & Stenström, T.A. (manuscript). Validation of Salmonella Typhimurium phage 28B as a thermoresistant indicator organism to evaluate the sanitizing effect of different treatment systems of organic waste.

Hurst, C.J., Gerba, C.P. & Cech, I. (1980). Effects of environmental variables and soil characteristics on virus survival in soil. *Applied and Environmental Microbiology* 40(6), pp. 1067-79.

Husman, A.M.d.R., Bijkerk, P., Lodder, W., Berg, H.v.d., Pribil, W., Cabaj, A., Gehringer, P., Sommer, R. & Duizer, E. (2004). Calicivirus inactivation by nonionizing (253.7-nanometer-wavelength UV) and ionizing (gamma) radiation. *Applied and Environmental Microbiology,* 70(9), pp. 5089-93.

Höglund, C., Ashbolt, N., Stenström, T.-A. & Svensson, L. (2002). Viral persistence in Source-Separated Human Urine. *Advances in Environmental Research*, 6, pp. 265-75.

Isbarn, S., Buckow, R., Himmelreich, A., Lehmacher, A. & Heinz, V. (2007). Inactivation of avian influenza virus by heat and high hydrostatic pressure. *Journal of Food Protection*, 70(3), pp. 667-73.

Jean, J., Morales-Rayas, R., Anoman, M.N. & Lamhoujeb, S. (2011). Inactivation of hepatitis A virus and norovirus surrogate in suspension and on food-contact surfaces using pulsed UV light (pulsed light inactivation of food-borne viruses). *Food Microbiology,* 28(3), pp. 568-572.

Jeffrey, D.J. (1995). Chemicals used as disinfectants: active ingredients and enhancing additives. *Revue scientifique et technique (International Office of Epizootics)*, 14, pp. 57-74.

Johansson, M., Emmoth, E., Salomonsson, A.C. & Albihn, A. (2005). Potential risks when spreading anaerobic digestion residues on grass silage crops - survival of bacteria, moulds and viruses. *Grass and Forage Science*, 60(2), pp. 175-85.

Jones, T.H. & Johns, M.W. (2012). Assessment of F-RNA coliphage as a potential indicator of enteric virus contamination of hog carcasses. *Journal of Food Protection,* 75(8), pp. 1492-1500.

Jurkiewicz, E., Villas-Boas, M., Silva, J. L., Weber, G., Hunsmann, G., & Clegg, R. M. (1995). Inactivation of simian immunodeficiency virus by hydrostatic pressure. *Proceedings of the National Academy of Sciences U S A*, 92(15), pp. 6935-37.

Kabell, S., Handberg, K. & Jrgensen, P.H. (2009). Inactivation of avian influenza virus H5N2 in acidified chicken pulp. *Veterinary Record,* 164(17), pp. 532-533.

Kasorndorkbua, C., Opriessnig, T., Huang, F. F., Guenette, D.K., Thomas, P.J., Meng, X.J. & Halbur, P.G. (2005). Infectious swine hepatitis E virus is present in pig manure storage facilities on United States farms, but evidence of water contamination is lacking. *Applied and Environmental Microbiology*, 71(12), pp. 7831-37.

Keeler S.P., Dalton M.S., Cressler A. M., Berghaus R. D. & Stallknecht D. E. (2014). Abiotic factors affecting the persistence of avian influenza virus in surface waters of waterfowl habitats. *Applied and Environmental Microbiology*, 80(9), pp. 2910-7.

Kingsley, D.H. (2013). High pressure processing and its application to the challenge of virus-contaminated foods. *Food and Environmental Virology*, 5(1), pp. 1-12.

Kingsley, D.H., Holliman, D.R., Calci, K.R., Chen, H.Q. & Flick, G.J. (2007). Inactivation of a norovirus by high-pressure processing. *Applied and Environmental Microbiology*, 73(2), pp. 581-585.

Koch, G. (2013). *FLURESIST-Studies on Avian Influenza Virus Survival in Poultry Commodities, Poultry Manure and the Environment.* (Sixth Framework Programme Priority 8.1. Periodic Activity Report): Call Identifier Fp6–2005–Ssp-5b-Influenza.

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Archiv für experimentelle Pathologie und Pharmacologie*, 162, pp. 480-483.

Leon, J.S., Kingsley, D.H., Montes, J.S., Richards, G.P., Lyon, G.M., Abdulhafid, G.M., Seitz, S.R., Fernandez, M.L., Teunis, P.F., Flick, G.J. & Moe, C.L. (2011). Randomized, double-blinded clinical trial for human norovirus inactivation in oysters by high hydrostatic pressure processing. *Applied and Environmental Microbiology*, 77(15), pp. 5476-82.

Ley, V., Higgins, J. & Fayer, R. (2002). Bovine enteroviruses as indicators of fecal contamination. *Applied and Environmental Microbiology*, 68(7), 3455-3461.

Li, X., Chen, H. & Kingsley, D.H. (2013). The influence of temperature, pH, and water immersion on the high hydrostatic pressure inactivation of GI.1 and GII.4 human noroviruses. *International Journal of Food Microbiology*, 167(2), pp. 138-43.

Lilleengen, K. (1948). Typing of Salmonella typhimurium by means of bacteriophage. *Royal veterinary College, Stockholm*.

Lima, S. M. , Vaz, A. C., Souza, T. L., Peabody, D. S., Silva, J. L. & Oliveira, A. C. (2006). Dissecting the role of protein-protein and protein-nucleic acid interactions in MS2 bacteriophage stability. *FEBS Journal*. 273(7), pp. 1463-75.

Lindahl, T. (1993). *Nature*. Instability and decay of the primary structure of DNA. *Nature*, 362(6422), pp. 709-15.

Linné, T., Emmoth, E., Klingeborn, B., McNeilly, F. & Allan, G. Presence of porcine circovirus type 2 in the swedish pig population. In: *Proceedings of 5th Nordic Virus Symposium ESCV Winter Meeting*, Stockholm, February 3-5 2000, p. 3.

Lu, H., Castro, A.E., Pennick, K., Liu, J., Yang, Q., Dunn, P., Weinstock, D. & Henzler, D. Survival of avian influenza virus H7N2 in SPF chickens and their environments. In: *Proceedings of Proceedings of the Fifth International Symposium on Avian Influenza*, Georgia, USA, 14-17 April, 2002.2003. pp. 1015-21.

Lund, B., Jensen, V.F., Have, P. & Ahring, B.K. (1996). Inactivation of virus during anaerobic digestion of manure in laboratory scale biogas reactors. *Antonie van Leeuwenhoek,* 69(1), pp. 25-31.

Martella, V., Lorusso, E., Banyai, K., Decaro, N., Corrente, M., Elia, G., Cavalli, A., Radogna, A., Costantini, V., Saif, L.J., Lavazza, A., Trani, L.d. & Buonavoglia, C. (2008). Identification of a porcine calicivirus related genetically to human sapoviruses. *Journal of Clinical Microbiology*, 46(6), pp. 1907-13.

Martens, W. & Bohm, R. (2009). Overview of the ability of different treatment methods for liquid and solid manure to inactivate pathogens. *Bioresource Technology*, 100(22), pp. 5374-8.

Mattison, K., Karthikeyan, K., Abebe, M., Malik, N., Sattar, S.A., Farber, J.M. & Bidawid, S. (2007). Survival of calicivirus in foods and on surfaces: Experiments with feline calicivirus as a surrogate for norovirus. *Journal of Food Protection*, 70(2), pp. 500-503.

Maunula L., Kaupke A., Vasickova P., Söderberg K., Kozyra I., Lazic S., van der Poel W.H., Bouwknegt M., Rutjes S., Willems K.A., Moloney R., D'Agostino M., de Roda Husman A.M., von Bonsdorff C.H., Rzeżutka A., Pavlik I., Petrovic T. & Cook N. 2013. Tracing enteric viruses in the European berry fruit supply chain. *International Journal of Food Microbiology*, 167(2), pp. 177-85.

McNulty, M. S. (1991). Chicken anaemia agent: a review. *Avian Pathology*, 20(2), pp. 187-203.

Mebus, C., Arias, M., Pineda, J. M., Taiador, J., House, C. & Sanchez-Vizcaino, J. M. (1997). Survival of several porcine viruses in different Spanish dry-cured meat products. *Food Chemistry*, 59(4), pp. 555-59.

Meng, X.J. (2010). Hepatitis E virus: animal reservoirs and zoonotic risk. *Veterinary Microbiology*, 140(3-4), pp. 256-65.

Meng, X.J. (2011). From barnyard to food table: The omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. *Virus Research*. 161(1):23-30.

Meng, X.J., Shivaprasad, H.L. & Payne, C. (2008). Avian Hepatitis E Virus In: Saif, Y.M. (ed. *Diseases of Poultry*. 12. ed. Iowa State Press, Ames, IA: Blackwell Publishing, pp. 441-448.

Mengeling, W.L. (1989). Porcine parvovirus. In: Pensaert, M.B. (ed. *Virus Infections of Porcines*. (Virus Infections of Vertebrates. Amsterdam: Elsevier Science Publishers.

Mesquita, M.M.F. & Emelko, M.B. (2012). Bacteriophages as Surrogates for the Fate and Transport of Pathogens in Source Water and in Drinking Water Treatment Processes, *Bacteriophages*. Dr. Ipek Kurtboke (ed.) InTech.

Middleton, D. J., Westbury, H. A., Morrissy, C. J., van der Heide, B. M., Russell, G. M., Braun, M. A. & Hyatt, A. D. (2002). Experimental Nipah virus infection in pigs and cats. *Journal of Comparative Pathology*. 126(2-3), pp. 124-36.

Monteith, H.D., Shannon, E.E. & Derbyshire, J.B. (1986). The inactivation of a bovine enterovirus and a bovine parvovirus in cattle manure by anaerobic

digestion, heat treatment, γ irradiation, ensilage and composting. *Journal of Hygiene*, 97(1), pp. 175-184.

Mormann S., Heißenberg C., Pfannebecker J., & Becker B. (2015). Tenacity of human norovirus and the surrogates feline calicivirus and murine norovirus during long-term storage on common nonporous food contact surfaces. *Journal of Food Protection*, 78(1),224-9

Munoz, O., De Nardi, M., van der Meulen, K., van Reeth, K., Koopmans, M., Harris, K., von Dobschuetz, S., Freidl, G., Meijer, A., Breed, A., Hill, A., Kosmider, R., Banks, J., Stark, K.D., Wieland, B., Stevens, K., van der Werf, S., Enouf, V., Dauphin, G., Dundon, W., Cattoli, G. & Capua, I. (2015). Genetic Adaptation of Influenza A Viruses in Domestic Animals and Their Potential Role in Interspecies Transmission: A Literature Review. *Ecohealth*. PMID: 25963341

Nazir, J., Haumacher, R., Ike, A. C. & Marschang, R. E. (2011). Persistence of avian influenza viruses in lake sediment, duck feces, and duck meat. *Applied and Environmental Microbiology*, 77(14), pp. 4981-5

Newman, J. F. & Brown, F. (1997). Foot-and-mouth disease virus and poliovirus particles contain proteins of the replication complex. *Journal of Virology*, 71(10), pp. 7657-62.

Okamoto, H. (2013). Culture systems for hepatitis E virus. *Journal of Gastroenterology*, 48(2), pp. 147-58.

Ottoson, J. (2005). *Comparative analysis of pathogen occurrence in wastewater – management strategies for barrier function and control.* Diss. Stockholm. Royal Institute of Technology (KTH). Swedish Institute for Infectious Disease Control (SMI).

Pesaro, F., Sorg, I. & Metzler, A. (1995). In situ inactivation of animal viruses and a coliphage in nonaerated liquid and semiliquid animal wastes. *Applied and Environmental Microbiology*, 61(1), pp. 92-7.

Pillai, S.P., Saif, Y.M. & Lee, C.W. (2010). Detection of influenza A viruses in eggs laid by infected turkeys. *Avian Diseases*, 54(2), pp. 830-3.

Poel, W.H.M.v.d., Vinje, J., Heide, R.v.d., Herrera, M.I., Vivo, A. & Koopmans, M.P.G. (2000). Norwalk-like calicivirus genes in farm animals. *Emerging Infectious Diseases*, 6(1), pp. 36-41.

Pontes L., Cordeiro Y., Giongo V., Villas-Boas M., Barreto A., Araújo J. R., Silva J. L. (2001). Pressure-induced formation of inactive triple-shelled rotavirus particles is associated with changes in the spike protein Vp4. *Journal of Molecular Biology*, 307(5), pp. 1171-9.

Popat, S.C., Yates, M.V. & Deshusses, M.A. (2010). Kinetics of inactivation of indicator pathogens during thermophilic anaerobic digestion. *Water Research*, 44(20), pp. 5965-72.

Rajkovic, A., Smigic, N. & Devlieghere, F. (2010). Contemporary strategies in combating microbial contamination in food chain. *International Journal of Food Microbiology*, 141(Suppl. 1), pp. S29-S42.

Richter, H.E. & Loewen, P.C. (1982). Rapid inactivation of bacteriophage T7 by ascorbic acid is repairable. *Biochimica et Biophysica Acta*, 697(1), pp. 25-30.

Roberts, P. & Hope, A. (2003). Virus inactivation by high intensity broad spectrum pulsed light. *Journal of Virological Methods*, 110(1), pp. 61-5.

Ryan, E., Mackay, D. & Donaldson, A. (2008). Foot-and-mouth disease virus concentrations in products of animal origin. *Transboundary and Emerging Diseases.* 55(2), pp. 89-98.

Salo, R.J. & Cliver, D.O. (1978). Inactivation of enteroviruses by ascorbic acid and sodium bisulfite. *Applied and Environmental Microbiology*, 36(1), pp. 68-75.

Scheinemann, H.A., Dittmar, K., Stöckel, F.S., Müller, H. & Krüger, M.E. (2015). Hygienisation and nutrient conservation of sewage sludge or cattle manure by lactic acid fermentation. *PLoS ONE,* 10(3) e0118230.

Sellers, R.F. Absolute safety. [Inactivation of viruses in animal effluents]. In: Proceedings of Communicable diseases resulting from storage, handling, transport and landspreading of manures. Proceedings of a CEC Workshop held at Hannover, 4-6 November 19801982. pp. 239-250.

Sharma, M., Shearer, A.E.H., Hoover, D.G., Liu, M.N., Solomon, M.B. & Kniel, K.E. (2008). Comparison of hydrostatic and hydrodynamic pressure to inactivate foodborne viruses. *Innovative Food Science & Emerging Technologies*, 9(4), pp. 418-422.

Shukla, P., Nguyen, H.T., Torian, U., Engle, R.E., Faulk, K., Dalton, H.R., Bendall, R.P., Keane, F.E., Purcell, R.H. & Emerson, S.U. (2011). Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. *Proceedings of the National Academy of Sciences U S A*, 108(6), pp. 2438-43.

Sigstam, T., Gannon, G., Cascella, M., Pecson, B. M., Wigginton, K. R. & Kohn, T. (2013). Subtle differences in virus composition affect disinfection kinetics and mechanisms. *Applied and Environmental Microbiology*, 79(11), pp. 3455-67.

Silva J. L. & Weber G. (1988). Pressure-induced dissociation of brome mosaic virus. *Journal of Molecular Biology*. 199(1), 149-59.

Silva J. L., Luan P., Glaser M., Voss E. W., Weber G. (1992). Effects of hydrostatic pressure on a membrane-enveloped virus: high immunogenicity of the pressure-inactivated virus. *Journal of Virology*, 66(4) pp. 2111-7.

Sinclair, R. G., Rose, J. B., Hashsham, S. A., Gerba, C. P. & Haas, C. N. (2012). Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Applied and Environmental Microbiology*. 78(6), 1969-77.

Smith, G.J.D., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S., Cheung, C., Raghwani, J., Bhatt, S., Peiris, J.S.M., Guan, Y. & Rambaut, A. (2009). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature (London),* 459(7250), pp. 1122-25.

Soukup, G. A. & Breaker, R. R. (1999). Relationship between internucleotide linkage geometry and the stability of RNA. *Rna.* 5(10), pp. 1308-25.

Spickler, A.R., Trampel, D.W. & Roth, J.A. (2008). The onset of virus shedding and clinical signs in chickens infected with high-pathogenicity and low-

pathogenicity avian influenza viruses. *Avian Pathology*, 37(6), pp. 555-77.

Straube, J., Albert, T., Manteufel, J., Heinze, J., Fehlhaber, K. & Truyen, U. (2011). In vitro influence of D/L-lactic acid, sodium chloride and sodium nitrite on the infectivity of feline calicivirus and of ECHO virus as potential surrogates for foodborne viruses. *International Journal of Food Microbiology*, 151(1), pp. 93-97.

Straube, J., Manteufel, J., Heinze, J., Fehlhaber, K., Truyen, U. & Albert, T. (2010). Low pathogenic avian influenza viruses (H3N8, H5N6): In vitro influence of D,L-lactic acid and sodium chloride on infectivity and virus persistence in short fermented raw poultry sausage. *Food and Environmental Virology*, 2(2), pp. 74-82.

Sun, J., Huang, L., Wei, Y., Wang, Y., Chen, D., Du, W., Wu, H. & Liu, C. (2015). Prevalence of emerging porcine parvoviruses and their co-infections with porcine circovirus type 2 in China. *Archives of Virology*, 160(5), 1339-44.

Surjit, M., Jameel, S. & Lal, S.K. (2004). The ORF2 protein of hepatitis E virus binds the 5' region of viral RNA. *Journal of Virology*, 78(1), ss. 320-8.

Sutton, D., Aldous, E.W., Warren, C.J., Fuller, C.M., Alexander, D.J. & Brown, I.H. (2013). Inactivation of the infectivity of two highly pathogenic avian influenza viruses and a virulent Newcastle disease virus by ultraviolet radiation. *Avian Pathology*, 42(6), pp. 566-568.

SVA (2015a) http://www.sva.se/djurhalsa/epizootier/newcastlesjuka

Swayne, D.E. & Beck, J.R. (2004). Heat inactivation of avian influenza and Newcastle disease viruses in egg products. *Avian Pathology*, 33(5), pp. 512-18.

Swayne, D.E. & Halvorson, D.A. (2008). Influenza. In: Saif, Y.M. (ed. *Diseases of Poultry*. 12. ed. Iowa State Press, Ames, IA: Blackwell Publishing, pp. 153-84.

Takahashi, M. & Okamoto, H. (2014). Features of hepatitis E virus infection in humans and animals in Japan. *Hepatology Research*, 44(1), pp. 43-58.

Tang, Q., Li, D., Xu, J., Wang, J., Zhao, Y., Li, Z., et al. (2010). Mechanism of inactivation of murine norovirus-1 by high pressure processing. *International Journal of Food Microbiology*, 137(2-3), pp. 186-89.

Terregino, C., Beato, M.S., Bertoli, E., Mancin, M. & Capua, I. (2009). Unexpected heat resistance of Italian low-pathogenicity and high-pathogenicity avian influenza A viruses of H7 subtype to prolonged exposure at 37 degrees C. *Avian Pathology*, 38(6), pp. 519-22.

Tree, J.A., Adams, M.R. & Lees, D.N. (2005). Disinfection of feline calicivirus (a surrogate for Norovirus) in wastewaters. *Journal of Applied Microbiology*, 98(1), pp. 155-162.

Turner, C. & Burton, C.H. (1997). The inactivation of viruses in pig slurries: a review. *Bioresource Technology*, 61(1), pp. 9-20.

Turner, C. & Williams, S.M. (1999). Laboratory-scale inactivation of African swine fever virus and swine vesicular disease virus in pig slurry. *Journal of Applied Microbiology*, 87(1), pp. 148-57.

Turner, C., Williams, S.M. & Wilkinson, P.J. (1999). Recovery and assay of African swine fever and swine vesicular disease viruses from pig slurry. *Journal of Applied Microbiology*, 87(3), pp. 447-53.

USEPA (2005). *Detecting and Mitigating the Environmental Impact of Fecal Pathogens Originating from Confined Animal Feeding Operations*: Review. Washington D. C.: USEPA.

Van der Poel, W.H. (2014). Food and environmental routes of Hepatitis E virus transmission. *Current Opinion in Virology*, 4C, pp. 91-96.

Ward, R.L. (1978). Mechanism of poliovirus inactivation by ammonia. *Journal of Virology*, (26)2, pp. 299-305.

Ward, R.L. & Ashley, C.S. (1977). Identification of the virucidal agent in wastewater sludge. *Applied and Environmental Microbiology*, 33(4), pp. 860-4.

Warren, J. (ed.) (1965). *Encephalomyocarditis Viruses*. Viral and Rickettsial Infections of Man. Philadelphia, Pa: Lippincott.

Warren, K.S. (1962). Ammonia toxicity and pH. *Nature*, 195, pp. 47-9.

Vasickova, P., Dvorska, L., Lorencova, A. & Pavlik, I. (2005). Viruses as a cause of foodborne diseases: a review of the literature. *Veterinarni Medicina*, 50(3), pp. 89-104.

Vasickova, P. , Pavlik, I., Verani, M. & Carducci, A. (2010). Issues concerning survival of viruses on surfaces. *Food and Environmental Virology*. 2(1), pp. 24-34.

Weeks, K. M. (2010). Advances in RNA structure analysis by chemical probing. *Current Opinion in Structural Biology*, 20(3), pp. 295-304.

Wekerle, J. Agricultural use of sewage sludge as a vector for transmission of viral disease. In: Proceedings of Epidemiological studies of risks associated with the agricultural use of sewage sludge: knowledge and needs1986. pp. 106-122.

Verdaguer, N., Jimenez-Clavero, M. A., Fita, I. & Ley, V. (2003). Structure of swine vesicular disease virus: mapping of changes occurring during adaptation of human coxsackie B5 virus to infect swine. *Journal of Virology*, 77(18), pp. 9780-9.

WHO (2006). *Review of latest available evidence on potential transmission of avian influenza (H5N1) through water and sewage and ways to reduce the risks to human health*. Geneva.

Widen, F., Sundqvist, L., Matyi-Toth, A., Metreveli, G., Belak, S., Hallgren, G. & Norder, H. (2011). Molecular epidemiology of hepatitis E virus in humans, pigs and wild boars in Sweden. *Epidemiology and Infection*, 139(3), pp. 361-371.

Wigand, R., Bachmann, P. & Brandner, G. (1981). Stabilization of isometric DNA viruses against thermoinactivation by lowered ionic strength. *Archives of Virology*, 69(1), pp. 61-69.

Wigginton K. R., Menin L., Sigstam T., Gannon G., Cascella M., Hamidane H. B., Tsybin Y. O., Waridel P. & Kohn T. (2012). UV radiation induces genome-mediated, site-specific cleavage in viral proteins. *ChemBioChem*, 13(6), pp. 837-45.

Wigginton K. R., Pecson B. M., Sigstam T., Bosshard F. & Kohn T. (2012). Virus inactivation mechanisms: impact of disinfectants on virus function and structural integrity. *Environmental Science and Technology*, 46(21), pp. 12069-78.

Vimont, A., Fliss, I. & Jean, J. (2015). Efficacy and mechanisms of murine norovirus inhibition by pulsed-light technology. *Applied and Environmental Microbiology*, 81(8), pp. 2950-7.

Vinneras, B. Comparison of composting, storage and urea treatment for sanitising of faecal matter and manure. In: *Proceedings of 11th International Conference of the FAO ESCO-RENA Network on Recycling Agricultural, Municipal and Industrial Residues in Agriculture (RAMIRAN), Murcia, Spain, 6-9 October 2004*.2007, pp. 3317-21.

Vinneras, B., Nordin, A., Niwagaba, C. & Nyberg, K. (2008). Inactivation of bacteria and viruses in human urine depending on temperature and dilution rate. *Water Research*, 42(15), pp. 4067-74.

Vinneras, B., Samuelson, A., Emmoth, E., Nyberg, K.A. & Albihn, A. (2012). Biosecurity aspects and pathogen inactivation in acidified high risk animal by-products. *J Environ Sci Health A Tox Hazard Subst Environ Eng*, 47(8), pp. 1166-72.

Vinnerås, B., Holmqvist, A., Bagge, E., Albihn, A. & Jönsson, H. (2003). The potential for disinfection of separated faecal matter by urea and by peracetic acid for hygienic nutrient recycling. *Bioresource Technology*, 89(2), pp. 155-61.

Wobus, C. E., Thackray, L. B., & Virgin, H. W. t. (2006). Murine norovirus: a model system to study norovirus biology and pathogenesis. *Journal of Virology*, 80(11), 5104-5112.

Vong, S., Ly, S., Mardy, S., Holl, D. Buchy, P. (2008). Environmental contamination during influenza A virus (H5N1) outbreaks, Cambodia, 2006. *Emerging Infectious Diseases*, 14(August 8), pp. 1303-1305.

Yates, M.V., Gerba, C.P. & Kelley, L.M. (1985). Virus persistence in groundwater. *Applied And Environmental Microbiology* 49(4), pp. 778-81.

Yazaki, Y., Mizuo, H., Takahashi, M., Nishizawa, T., Sasaki, N., Gotanda, Y. & Okamoto, H. (2003). Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. *Journal of General Virology*, 84(9), pp. 2351-57.

Zou, S., Guo, J., Gao, R., Dong, L., Zhou, J., Zhang, Y., Dong, J., Bo, H., Qin, K. & Shu, Y. (2013). Inactivation of the novel avian influenza A (H7N9) virus under physical conditions or chemical agents treatment. *Virol Journal*, 10, p. 289.

Zuber, S., Butot, S. & Baert, L. (2013). Effects of treatments used in food processing on viruses. In: Frans J.M. Smulders, B.N., Herbert Budka (ed. *Foodborne viruses and prions and their significance for public health* Food safety assurance and veterinary public health no. 6). Wageningen: Wageningen Academic Publishers, pp. 113-136.

Acknowledgements

The studies performed in this thesis was performed at SVA, and was financed by the research programme Organic Waste-Resource or Risk in Sustainable Agriculture (PROWARR), Swedish University of Agricultural Sciences (SLU) and the Swedish Board of Agriculture (Paper I); European project Fluresist (SS PE-CT-2006-44311), Swehatch Flyinge AB (Paper II and III); Pathogen Combat-EU Food-CT-2005.007181 Project and NC Hyperbaric, Spain (Paper IV).

I wish to express my sincere gratitude for supporting this work to Professor Anders Engvall and Dr. Jens Mattsson, the former and present director-general of SVA. The former and present Head of Department of Biomedical Sciences and Veterinary Public Health, SLU, Professor Martin Wierup and Professor Leif Norrgren, former Head of Department of Wildlife, Fish and Environment, SVA are thanked for supporting this thesis.

I thank also the former and present Heads of Department of Virology, Immunobiology and Parasitology (VIP), SVA, Professor and former main supervisor Berndt Klingeborn, Associate Professor Jean-François Valarcher and Professor and Adjunct Professor at Uppsala University (UU) Kjell-Olov Grönvik, for the possibility to research. For providing further resources, the former and present Heads of Department of Chemistry, Environment and Feed Hygiene (KMF), SVA, Professor and Adjunct Professor at UU Ulf Bondesson and Associate and Visiting Professor at UU, Mikael Hedeland are thanked. The support of Dr Lena Renström, former Head of Section for Disease Control and Virus Validation and present Head of Section of Virology – diagnostics and preparedness and former and present Heads of Section for Research and development, VIP, Dr Mikael Juremalm and Associate Professor Jan Chirico, is genuinely appreciated.

I am in great dept to my supervisors, for supporting me thoroughly so I could pursue this thesis:

Main supervisor Associate Professor Jakob R. Ottoson, for your guidance, patience and invaluable help and encouragement to make this thesis possible. I have really learnt a lot through the course of this thesis. Thank you also for nice travel company to Fluresist meetings.

My former main supervisor and co-supervisor Professor Sándor Belák, for your support and viral knowledge, and for introducing me to new methods in the virus field. The well planned study trip regarding veterinary virology was greatly appreciated.

Co-supervisor Professor and Adjunct Professor, SLU, Ann Albihn, for your ideas and help in initiating the virus studies, and your continuous support. Thank you also for the great planning days with the group, always with nice lunches at your house.

Co-supervisor Associate Professor Björn Vinnerås, for your support and providing the opportunity to get aquainted with ammonia disinfection regarding viruses. Also, thank you for your company and help at Ramiran meetings.

Co-supervisor Associate Professor Frederik Widén, for your support and the opportunity to learn more about Hepatitis E virus, and work with the models. Thank you for your nice travel company to the PC meetings regarding this project.

I would like to thank others who have contributed to this work:

Dr Josefine Elving, co-author, for all your help with everything from the thesis to work in the summerhouse, and nice travel company.

My coauthors Dr Leena Sahlström, Dr Elisabeth Bagge, Dr Annika Holmqvist, and Professor Marie-Louise Danielsson-Tham for fruitful collaboration.

Irene Dergel, room mate, for collaboration and nice company on the work trips, and for expertise with cells and all the PD10-runs.

Professor Jordi Rovira, Dr Elena Corcuera and Dr Diego Wilches, for collaboration in the high pressure experiments and a very pleasant time in Burgos.

Professor Andreja Rajkovic for providing equipment and advice for the intense light pulse treatment, and great hospitality in Ghent.

Dr Giorgi Metreveli for appreciated encouragement of the thesis work and nice collaboration in different virus projects.

Yvonne Hallén and Gun-Britt Rydén for expert help with computer work and figure layouts, Mary MaAfee for English revision of the manuscripts, and Dimitris Papageorgiou, SLU, for help at the printing.

Kenneth Fermner and personnel at Lantmännen Swehatch AB for great help with the ammonia large scale study regarding hatchery waste.

Dr Siamak Zohari and Dr Jay Lin for answering a number of questions about influenza and hepatitis E virus regarding this thesis, Dr Annika Nordin regarding ammonia experiments, and Dr Jörgen Fidjeland for statistics help.

I would also like to thank:

Professor Tommy Linné, for the opportunity to start working with virus and allowing for a lot of nice activities together with you and Mikael, Annika, Anita, Helena, Bernt, Jorge and others.

For advice in virus validations I thank Bertil Eriksson and Eva Olsson, and in GLP matters Bo Sundqvist, Ulla Johansson och Suzanne Kjellberg.

Gunilla Blomqvist, Ulrika Allard Bengtsson and Cecilia Hultén for friendship and collaboration with virus inactivation and sanitation.

Alia, Mikael L., Misha, Lihong and Hongyan, for fruitful collaboration, nice chats and hospitality.

Berndts lab group at BMC, with Maud, Anna-Britta, Annette, Ann, Eva L., Maria-Elena och Susana, for nice friendship and good times.

The FoU group with former and present members, Gunnel, Åsa, Harri, Katarina, Ann-Sophie, Karin T., Karin A, Anna L., Anna A., Sevinc, Sara F., Boel, Eva O., Robert, Bo, Mats, Karin U., Eva W., Cecilia, Helena, Pia, Claudia, Vinay, Tobias, Anders, Shaman, Hari, Mehdi.

My former section of diagnostics with former and present heads and members Louise, Annie, Anna-Karin, Jane, Eva Ek., Gunilla, Jolanta, Mia, Anna, Tina, Fahkri, Karin T., Karin v. S., Ulla, Kerstin, Linda, and especially Ing-Britt and Caroline for cell cultures.

Former SMS group members Karin, Gunnar, Emelie, Anna, Erik, Mare and Jörgen, and earlier members Ingela and Mats.

For help and support, I thank Annica, especially for all help during the lic thesis, Birgitta, Anki and Maria, and Berka for his helpfulness with technical matters.

For very nice music activities I thank spelgruppen Freespel and other musicians on nyckelharpa and other string instruments, and kören Rediviva.

For enjoyable dinners and travels, I thank Marianne, Annette, Kerstin, Gunnar, Birgitta, Christer, Ann, Cecilia and Marita.

A lot of other people have contributed to a nice working athmosphere, and also other friends outside work, even if you are not mentioned, you know who you are.

For always being there, my special thanks to my family, father, sister and brother with families, uncle, and especially my children Anton and Anna, you are in my heart.