Thermal Treatment of Organic Waste and its Function as a Controlled Risk Mitigation Strategy

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Abstract

Land application of organic waste is a convenient disposal method, improves soil properties by adding organic matter and recycles plant nutrients. Used in this way, organic waste is a resource, but it can also be a transmission route for pathogens with animal and human origin, such as *Salmonella*, verotoxin-producing *Escherichia coli* (VTEC) and avian influenza virus (AIV).

Many pathogens are known to be able to survive for long time periods in soil after land application of organic waste. The total amount of pathogens entering the environment is eventually reduced due to environmental factors, *e.g.* UV-light and desiccation. Mitigation strategies applied after land application, for instance holding times, can reduce the risk for disease transmission to grazing animals. However, it is difficult to predict the reduction occurring within a given time. Thus, controlled mitigation strategies, such as, sanitization treatments are therefore needed.

This thesis investigated the effect of thermal treatment on the inactivation rate of bacterial and viral pathogens, aiming to produce a hygienically safe end-product. For thermal treatment of organic waste it is recommended to keep a temperature above 50°C. Some organisms, such as AIV, can be rapidly reduced at lower temperatures. However, many organisms show a slow reduction at lower temperatures and some bacterial pathogens, such as *Salmonella*, might even regrow. The reduction target of 5 $\log_{10}$, for *Salmonella* Senftenberg W775 and *Enterococcus faecalis*, can be reached within 11.7 h at a temperature of 50.5°C. To reach a 3 $\log_{10}$ reduction of the thermotolerant virus, in this case porcine parvovirus, sufficiently longer treatment times are needed, *e.g.* 42 h at 55°C. However, the use of such thermotolerant viruses in the validation process has been questioned. Making use of the less thermotolerant bacteriophage ΦX174, as a process indicator, indicates that the treatment recommendations concerning viral reduction could be lowered to 5.8 h at 55°C.

Thermal treatment can ensure; that a hygienically safe end-product is obtained from organic waste, safe handling of material from AI outbreaks, and help in reducing the risk for pathogen transmission to the environment.

Keywords: Salmonella, enterococci, bacteriophage, AIV, parvovirus, manure, animal by-products, risk assessment, grazing animals.

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We must realize that the familiar plants and animals are not the only living forms on the earth, but, on the contrary, there are all about us everywhere great numbers of very tiny living creatures, too small to be seen by the naked eye.

Kenneth L. Burdon, Textbook of Microbiology, 3rd ed. 1950

Contents

List of Publications

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

I Elving, J., Vinnerås, B., Albihn, A., Ottoson, J. (2012). Thermal inactivation of pathogens to mitigate health risks from digestate and compost. In manuscript.

II Elving, J., Emmoth, E., Vinnerås, B., Albihn, A., Ottoson, J. (2012). Composting for elimination of Avian influenza virus. *Applied and Environmental Microbiology* 78, 3280-3285.

III Elving, J., Ottoson, J.R., Vinnerås, B., Albihn, A. (2010). Growth potential of faecal bacteria in simulated psychrophilic/mesophilic zones during composting of organic waste. *Journal of Applied Microbiology* 108(6), 1974-1981.

IV Elving, J., Nyberg, K., Albihn, A., Ottoson, J. (2012). Modelling cattle exposure to *Salmonella* in pasture after application of organic fertilisers. In manuscript.

The contribution of Josefine Elving to the papers included in this thesis was as follows:

I J. Elving, B. Vinnerås, A. Albihn and J.R. Ottoson planned the study and J. Elving carried out the experimental work with help from Erik Helmersson. J. Elving did the writing, with revision by co-authors.

II J. Elving, E. Emmoth, B. Vinnerås, A. Albihn and J.R. Ottoson planned the study. J. Elving and E. Emmoth carried out the experimental work. J. Elving did the writing, with revision by co-authors.

III J. Elving, J.R. Ottoson, B. Vinnerås and A. Albihn planned the study and J. Elving carried out the experimental work. J. Elving did the writing, with revision by co-authors.

IV J. Elving, K. Nyberg, A. Albihn and J.R. Ottoson planned the project. J. Elving and J.R. Ottoson planned and constructed the model for the QMRA and J. Elving did the writing, with contributions by co-authors.

Definitions

It is not unusual for definitions to vary between different studies. The following definitions were used in this thesis:

Animal by-products	Defined in Regulation (EC) No. 1069/2009, *e.g.* manure, food waste and slaughterhouse waste.
Biosolids	Solid or semisolid organic material obtained from treated wastewater, often used as a fertiliser or soil amendment
D-value	The amount of time required for a 1 $\log_{10}$ reduction (90%) in the number of organisms
Manure	Material consisting of faecal material in combination with bedding material, may also include urine and other body fluids
Mesophilic temperature	15-45°C
Model organism	An organism used to as a surrogate to model the behaviour of pathogenic organisms in *e.g.* the environment
Organic waste	Biodegradable waste, *e.g.* animal by-products and biosolids.
Pathogen	An organism capable of causing disease or illness in its host *e.g.* through infection or toxin production
Process indicator	An organism used to monitor the efficiency of a treatment process
Psychrophilic temperature	<15°C
Risk analysis	A process consisting of three components: risk assessment, risk management and risk communication

Risk assessment	A scientifically based process consisting of: hazard identification, dose-response assessment, exposure assessment and risk characterization. Can be either quantitative or qualitative.
Thermophilic temperature	>45°C
Volatile solids	The fraction lost during heating at 550°C, a measure of the organic matter content of a material
Zoonoses	Diseases that can be transmitted between animals and humans
z-value	The number of °C required to change the D-value by a factor of 10

Abbreviations

ABP	Animal by-products
AIV	avian influenza virus
ATCC	American Type Culture Collection
B.	*Bacillus*
C.	*Campylobacter*
CCUG	Culture Collection, University of Gothenburg
CFU	Colony-forming unit
Cl.	*Clostridium*
HPAIV	highly pathogenic AIV
E.	*Escherichia*
EHEC	Enterohaemorrhagic *E. coli*
Ent.	*Enterococcus*
L.	*Listeria*
LPAIV	Low pathogenic AIV
MC	Moisture content
NCTC	National Collection of Type Cultures
PFU	Plaque-forming unit
PPV	Porcine parvovirus
QMRA	Quantitative microbial risk assessment
S.	*Salmonella*
spp.	Sub-species
$TCID_{50}$	50% Tissue Culture Infectious Dose
TS	Total solids
VTEC	Verotoxin-producing *E. coli*

1 Introduction

Land application of organic waste, for example manure, biosolids, slaughterhouse waste and food waste, serves several purposes. Not only is it a practical solution to waste handling, but it also contributes to recycling of valuable plant nutrients, such as, nitrogen, phosphorus, sulphur, and potassium, as well as some essential micro nutrients, *e.g.* nickel, zinc, and copper. Thus the use of organic waste as fertiliser works as a supplement to commercial fertilisers and serves to decrease the amounts required of the latter. Addition of organic matter to soils can also improve soil properties such as structure and water-holding capacity, making conditions more favourable for root growth and increasing the drought tolerance of the vegetation (Williams *et al.*, 1995; Lavelle, 1988; Oades, 1984). However, recycling of organic waste may also serve as a transmission route for pathogens, *i.e.* disease causing organisms, of animal and human origin to the environment and into the food chain (Albihn & Vinnerås, 2007). Many of these pathogens are zoonoses, and therefore of special interest concerning human and animal health.

It is possible recycle the plant nutrients in organic waste and manure without recycling pathogens through the use of mitigation strategies, *e.g.* sanitisation treatment, holding time and, crop restrictions. The risk for presence of pathogens in organic waste varies depending on the incoming material, for example, fractions such as garden waste does not pose as high a risk as slaughterhouse waste. Hence the composition of the incoming material has to be taken into consideration when evaluating the risk associated with recycling, as well as when and where the material is intended to be applied to land. Due to the possibly long survival time of pathogens in soil and vegetation after application of fertilisers to land, sanitisation treatment has been recognised as an important step in reducing the risk of pathogen transmission (Krause & Hendrick, 2011).

To estimate the consequences from exposure, of individuals or populations, to pathogens, a risk assessment approach can be used. This is done by combining the performance of different steps in a scenario and by reducing the impact of uncertainty in the prediction. The risk assessment is part of a concept known as risk analysis. Risk analysis include, risk assessment, risk management, and risk communication (WHO, 2007; Haas *et al.*, 1999).

This thesis deals with the survival, growth, and inactivation of pathogens in organic waste and the establishment of barriers to limit the risk of disease transmission associated with recycling of such materials.

2 Objectives

The focus of this thesis is on thermal sanitisation treatments for organic waste and manure and how such treatments can be used as barriers to minimise the transmission of pathogens. The overall aim of the thesis was to increase our understanding of how hygienically safe recycling of organic waste can be achieved, so as to allow recycling of plant nutrients without causing disease in humans and animals. Specific objectives were to:

- Determine the inactivation rate of *Salmonella* Senftenberg W775, *Enterococcus faecalis*, the ΦX174 phage and, porcine parvovirus (PPV) at temperatures ranging from 46-55°C, and investigate the possibility of these temperatures fulfilling the reduction targets for bacterial and viral reduction of 5 $\log_{10}$ and 3 $\log_{10}$ respectively

- Investigate the effectiveness of pasteurization at 70°C for 60 min in reducing *S.* Senftenberg W775, *Ent. faecalis* and PPV compared with the reduction targets mentioned above

- Evaluate the use of *Ent. faecalis* and PPV as process indicators for *Salmonella* spp. and viruses, respectively, in validation of thermal sanitisation treatments

- Determine the effectiveness of thermal treatment in reducing avian influenza virus

- Investigate the regrowth potential of *Salmonella* and model organisms in material from organic waste composts

- Determine the relative effect of different barriers in prevention of disease transmission to grazing animals from fertilisation of arable land.

3 Background

Although the majority of microorganisms are harmless to humans and animals, some of them are capable of causing disease through infection or production of toxin. Pathogens may pass from person to person or animal to animal but also between human and animals *i.e.* zoonoses.
The relationship between the environment, host and pathogen can be illustrated by the epidemiology triangle (Figure 1). The three cornerstones of the epidemiology triangle are the agent/pathogen causing the disease, the host, and environmental factors. In this thesis the latter is primarily the matrix causing or allowing the disease to be transmitted. In establishing and modelling disease control strategies, knowledge about all three cornerstones of the triangle is important in order to get a good picture of the disease and the disease process. Understanding the three cornerstones is also important in risk analysis and when building risk models for pathogens.

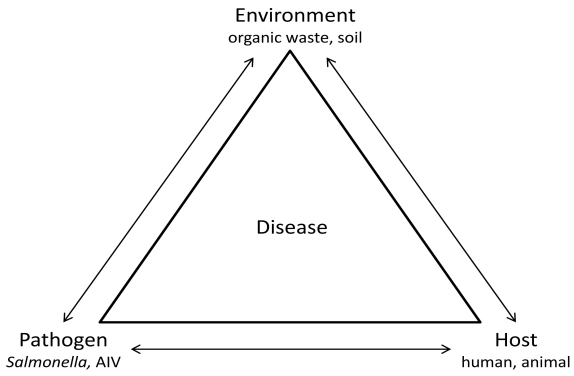

Figure 1. The epidemiology triangle.

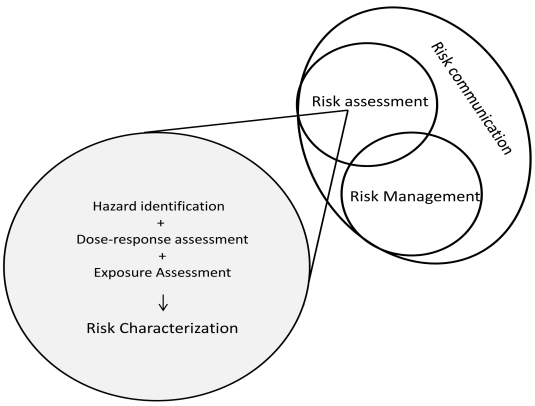

Figure 2. The components of a risk analysis according to Haas *et al.* (1999).

Risk analysis or risk-based decision making is used to estimate, evaluate and discuss the risk of adverse events and mitigation-processes. Risk analysis involves three processes; Risk Assessment, Risk Management and Risk Communication (Figure 2) and aims to manage public health hazards with a transparent approach (WHO, 2007; Haas *et al.*, 1999). This thesis does not deal with the entire risk analysis process, but focuses on science-based risk assessment and mitigation strategies to reduce the risk of disease transmission from organic waste as a basis for later use in risk management and risk communication.

Table 1. *Processes involved in a risk assessment (Haas et al., 1999)*

Hazard identification	Identifies relevant pathogens.
Dose-response assessment	Describes the relationship between dose and negative health effects in an population.
Exposure assessment	Estimates the frequency, amount and duration of exposure for relevant transmission routs.
Risk characterization	Addresses the questions asked by the risk manager and evaluates the risk management options by integration of the three previous steps. The process results in a risk estimate which can be on an individual or population basis, *e.g.* illness per serving of salad or total number of illness in a population in a year. Evaluates uncertainty and sensitivity. The hazard characterization is usually highly adaptable between different risk assessments for the same pathogen by taking into consideration the effect of different matrices.

The purpose of risk assessment is to estimate the risk associated with a hazard. Risk assessment includes: hazard identification, dose-response assessment and, exposure assessment, which all come together in risk characterisation (Table 1)(Haas *et al.*, 1999). The outcome of the risk assessment process is in turn used in formulation and implementation of measures to reduce the likelihood of an unwanted event occurring in the risk management process. The concept of "acceptable risk" plays a key role in risk management. Proposals of an "acceptable risk" limit should involve representatives from various parts of society. The term "acceptable risk" might need further explanation. Even if the goal is to eliminate the risk a zero risk is unachievable, hence the term acceptable risk. Defining when a risk is acceptable is not easy. A risk can, for instance, be seen as acceptable when it falls below a subjective defined probability or the level already tolerated; when the cost for reduction of the risk is higher than the costs saved, for example for animal production; and when public health professionals, the general public or politicians deem it to be acceptable (Hunter & Fewtrell, 2001). If the risk is considered to be too high, preventative measures should be taken to decrease the risk to an acceptable level.

3.1 Hazard identification

In the case of organic waste pathogens can originate from diseased or infected but symptom-free animal or human carriers. The microorganisms can be excreted with faeces or urine and other secretions, but may also be present within the tissues of infected animals or on faeces-contaminated tissues.

3.1.1 Pathogens in organic waste

In addition to infectious or toxin producing bacteria, virus and parasites, a number of pathogenic fungi have been found in organic waste, *e.g. Aspergillus fumigates* and *Candida* spp. (Haug, 1993). However, fungi mainly cause health problems via direct skin contact or inhalation and thus are of more concern for workers at plants handling organic waste than for people exposed to the recycled end-product. Many of the pathogens found in organic waste are zoonoses. Zoonoses are of special interest concerning organic waste recycling since they do not only have impact on public health, but also on livestock economies and wildlife. Some examples of such organisms are given in the following paragraphs.

Bacteria

Organic waste can contain bacteria able to cause infection and toxin-producing bacteria from several different genera, *e.g. Salmonella, Campylobacter, Listeria, Escherichia, Bacillus* and *Clostridium* (Strauch, 1991; Dudley *et al.*, 1980). Among these are pathogens capable of causing sub-clinical infections *i.e.* symptom-free infections, as well as serious infections.

Most *Salmonella* species are zoonotic but some are host-specific, *e.g. S.* Typhi and *S.* Parathypi, which only infect humans, and *S.* Pullorum and *S.* Gallinarum, which mainly cause disease among young poultry (SVA, 2008). A common route of transmission to humans is the faecal-oral route *i.e.* ingestion of the bacteria by consumption of, for example, contaminated meat, meat products, leafy greens, eggs and egg products (SVA, 2011; Herman *et al.*, 2008). Every year millions of cases of salmonellosis and thousands of human deaths caused by *Salmonella* are reported worldwide. Thus, salmonellosis is one of the most common and widely distributed foodborne diseases (WHO, 2005). In Sweden approximately 3,000-4,000 cases of human salmonellosis are reported every year, the majority of which are contracted abroad (SVA, 2011). Common symptoms of salmonellosis are diarrhoea, fever and vomiting. However, more serious complications can occur such as meningitis, sepsis and death, especially in sensitive populations, such as elderly and immune-compromised individuals (Saphra & Winter, 1957). It is not uncommon for animals to be asymptomatic. However, the infection can manifest in clinical symptoms such as diarrhoea, fever, dehydration, bloody faeces, abortion, poor general condition and death (SVA, 2011).The frequency of *Salmonella*-infected animals in Sweden is low. The expected herd-prevalence is approximately 3.7% in dairy herds (Ågren *et al.*, 2012) and sampling of carcasses of swine and broilers at slaughter has given negative results in recent years (Lindblad *et al.*, 2007; Lindblad *et al.*, 2006). This can be attributed to a strict Salmonella control programme in livestock.

Verotoxin-producing *E.coli* (VTEC) has the potential to cause gastrointestinal disease with serious symptoms such as bloody diarrhoea and haemolytic uremic syndrome (HUS) with renal failure. About 300 cases of EHEC are reported annually in Sweden. Half of the reported cases are contracted abroad (SVA, 2011). Enterohaemorrhagic *E.coli* (EHEC) is a subgroup of VTEC (Figure 3) that has the potential to cause haemorrhagic colitis in humans (Welinder-Olsson & Kaijser, 2005). The most common serotype in outbreak scenarios is serotype O157. Thus, *E. coli* O157 can be seen as the prototype organism for EHEC (Levine *et al.*, 1987). However, there is also other serotypes which can cause disease and not all O157 causes disease (Figure 3). Animals are often asymptomatic carriers of the bacteria, *i.e.* they do

not develop clinical disease. Several animal species can be infected by VTEC, but cattle have been identified as the main reservoir. Unpasteurized milk and apple cider, contaminated water and fresh produce such as leafy greens have been implicated as sources of infection (SVA, 2011; Bell *et al.*, 1994; Besser *et al.*, 1993). In Sweden, surveys have isolated *E. coli* O157 from 8.9% of cattle herds tested, although in southern Sweden the prevalence is as high as 23% (Eriksson *et al.*, 2005). Human disease has also been connected to contaminated recreational waters (Gyles, 2007).

Campylobacter is a common cause of bacterial gastroenteritis (Mead *et al.*, 1999). Campylobacteriosis occurs worldwide and approximately 5-14% of all diarrhoea worldwide is thought to be caused by *Campylobacter* spp. (Coker *et al.*, 2002). In Sweden, approximately 7000 cases of campylobacteriosis are reported each year, of which most are related to travelling outside Sweden. It is primarily the species *C. jejuni* and *C. coli* that are associated with disease in humans, although other species can also cause infection. The bacteria can be present in faecal material from animals and humans and are primarily transmitted through contaminated food, feed and water. Risk factors for infection in humans include consumption of poultry and contaminated raw milk and water (Friedman *et al.*, 2004; Kapperud *et al.*, 1992). In humans, the infection generally causes symptoms diarrhoea, abdominal pain, fever, headache and vomiting, but in sensitive groups in the population it can be fatal (SVA, 2011). Infection in animals is usually asymptomatic, but some animal species, especially sheep, can develop clinical disease when infected, *e.g.* neurological symptoms, abortion, mastitis or septicaemia.

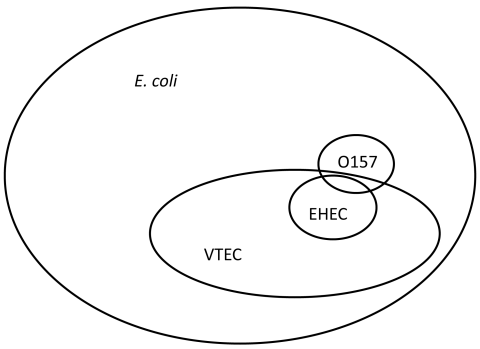

Figure 3. Schematic explanation of the relationship between commensal *E.coli,* VTEC, EHEC and serotype O157 (S. Boqvist).

Listeriosis is a zoonosis, caused by the bacteria *Listeria monocytogenes*. The infection is usually symptom-free, although infections manifesting symptoms do occur in some sensitive groups in the population such as pregnant women and immunocompromised individuals (Cork & Checkley, 2011). Bacteria belonging to the *Listeria* spp. are present in the gastrointestinal tract of mammals and in the environment, where they have been isolated from soil, plants, water and sewage (Mitscherlich & Marth, 1984). *Listeria* species have the ability to grow at temperatures ranging from 1 to 45°C (Junttila *et al.*, 1988). The ability to grow at low temperatures affects the food-borne transmission of the bacteria, since they are able to multiply at refrigerator temperatures. As a result of this, the main sources are contaminated food such as smoked or marinated vacuum-packed fishery products or other product with a long shelf-life (SVA, 2011). The number of listeriosis cases is increasing and, in Sweden approximately 60-70 cases are reported every year (SVA, 2011).

Whereas most bacteria are unable to survive under harsh conditions sporulating bacteria such as *Bacillus* spp. and *Clostridium* spp. have developed defence mechanisms that enable them to withstand difficult environments. Under extreme conditions, stress or nutrient deprivation, the vegetative cells sporulate to form an endospore, which is a highly resistant structure (Madigan & Martinko, 2006; Nicholson *et al.*, 2002; Nicholson *et al.*, 2000; Mitscherlich & Marth, 1984). Endospores are not metabolically active, but under favourable conditions they can germinate and develop into fully functional vegetative cells.

Most *Bacillus* spp. and *Clostridium* spp. are harmless, but species such as *B. anthracis, B. cereus, Cl. botulinum* and *Cl. perfringens* can cause disease in the host either by infection or production of toxins (Krause & Hendrick, 2011; Valdez *et al.*, 2009; Ozkocaman *et al.*, 2006; Gyles & Thoen, 1993). *B. cereus* and *Cl. perfringens* are both opportunistic pathogens, *i.e.* are usually harmless but can cause disease under particular circumstances and then often in sensitive groups in the population (Valdez *et al.*, 2009; Ozkocaman *et al.*, 2006; Gyles & Thoen, 1993). Other species such as *B. anthracis* and *Cl. botulinum* cause serious disease.

B. anthracis is the cause of anthrax, a zoonotic disease in humans and livestock. *B. anthracis* is primarily transmitted from infected or diseased animals and contaminated soil. Due to the resistance of the spore long survival times in soil has been recorded and thus contact with buried carcasses still has the potential to result in infection of animals and humans after decades (Mitscherlich & Marth, 1984). Depending on the route of transmission, the bacteria are able to cause a wide range of symptoms in humans, *e.g. pneumonia* and septicaemia. If ingested, the symptoms are usually abdominal

pain, fever, sickness, diarrhoea and vomiting, and may lead to septicaemia (Spencer, 2003). In animals, the symptoms vary between different animal species, but in sensitive species such as ruminants, the infection often lead to sudden death of the animal before signs of illness have been observed (Jones *et al.*, 1997).

Cl. botulinum is able to produce toxins, the best known being its neurotoxin, which causes botulism with paralysis in both humans and animals. The organisms are excreted in the faeces of infected animals and can be found in the environment. Transmission via ingestion of the toxin give rise to classical botulism, which is the most common form of botulism, leading to intoxication with following paralysis. *Cl. botulinum* can colonise the intestine when ingested as spores. Following colonisation the toxins are produced, leading to intoxication (Sobel, 2009). Botulism is not common in Sweden and since 1969 only around 10 human cases of botulism intoxication has been reported (SMI, 2012). Likewise, only a small number of cases of botulism in animals have been reported in Sweden (SVA, 2012). In animals ingestion of contaminated feed or contact with contaminated litter from infected or diseased animals can cause intoxication (Kennedy & Ball, 2011; Galey *et al.*, 2000).

Virus

Most viruses present in organic waste are not zoonotic. However, there are zoonotic viruses associated with organic waste that are of interest concerning human and animal health, for example, hepatitis E virus (HEV) and influenza A virus (Milinovich & Klieve, 2011; USEPA, 2005; Sobsey *et al.*, 2001).

Of the four genotypes of HEV, genotypes 1 and 2 have almost only been detected in humans, while serotypes 3 and 4 have been isolated from humans as well as other mammals, *e.g.* swine, wild boar, rabbits and deer, which may act as reservoirs for the virus (Takahashi *et al.*, 2004; Matsuda *et al.*, 2003; Khuroo *et al.*, 1995). HEV causes acute, generally mild, infections in humans and is primarily transmitted via the faecal-oral route *i.e.* via contaminated water and food (Khuroo & Khuroo, 2008; Goens & Perdue, 2004). However, hepatic failure does occur and in pregnant women the mortality rate is approximately 20% (Khuroo & Kamili, 2003; Mast & Krawczynski, 1996). Previously HEV was mainly a problem in endemic areas and was hence associated with travel to endemic areas. However, the number of reported cases of HEV genotypes 3 and 4, without a connection to travel to endemic areas, has increased recently (Dalton *et al.*, 2008). In Sweden, a limited number of human cases of HEV have been reported and most are thought to have contracted the virus abroad, primarily in Asia. Those infected within Sweden

and Europe were all infected with genotype 3. This is also the genotype found in domestic pigs and wild boars in Sweden (Widen *et al.*, 2011).

Wild birds are the reservoirs for low pathogenic viruses of avian influenza (LPAIV). LPAIV can mutate into highly pathogenic form (HPAIV) in poultry flocks (SVA, 2011). The low pathogenic form often causes asymptomatic infection or mild respiratory disease in animals, while HPAIV causes a wide range of clinical symptoms such as respiratory distress, diarrhoea and sudden death. AIV is thought to be transmitted at close range through bird secretions to human respiratory mucosa, or through gastrointestinal infection as a result of contaminated foods (Vong *et al.*, 2008; Brankston *et al.*, 2007). The HPAIV strain H5N1 has been associated with infection in humans, with a mortality rate of 60% (Gambotto *et al.*, 2008).

There are also several viruses that are classified as potential zoonoses, *e.g.* Rotaviruses. Although they are generally species-specific, cross-species transmission has been demonstrated to be possible (Midgley *et al.*, 2012).

3.1.2 Model organisms and process indicators

Some pathogens are not practical to analyse for different reasons, *e.g.* they can be too time consuming or expensive to analyse or associated with too great a human health risk. In particular in the large amount of samples needed during the validation of treatment methods and sampling associated with process controls may contribute to high analysis costs and further development of new sanitisation treatment options. As an alternative, model organisms can be used to study and model the behaviour of pathogens in the environment and during sanitisation treatments. A suitable model organism should preferably be non-pathogenic to humans, easy to analyse and equally or slightly more resistant than the pathogen or pathogens in question. When studying faecal-oral transmission, it is also an advantage if the organism is present in the intestine of warm-blooded animals, making it possible to study the survival and inactivation without inoculating the materials.

This thesis examines the use of various organisms to model the behaviour of pathogens during thermal treatment. By establishing such relationships, it might be possible to use naturally occurring microorganisms as models and process indicators. Once such a relationship has been established, the model organism can be used in treatment processes, as a process indicator, to ensure that the process is fulfilling the reduction targets set by current standards and regulations.

Some of the most commonly used bacterial model organisms belong to the *Enterococccaceae* and *Enterobacteriaceae* families *e.g.* coliforms, *E. coli* and enterococci. Enterococci have been reported to be generally more resistant than

coliforms to different environmental factors (Bitton, 1999) and have been shown to be able to survive thermal composting for a long time after organisms such as *Salmonella* spp. have been inactivated. They may therefore be seen as a conservative process indicator (Vinnerås, 2007; Craven *et al.*, 1997; Shuval *et al.*, 1991). However, bacterial model organisms such as coliforms and enterococci have been shown to be inadequate model organisms for several pathogens, especially for viruses and protozoa (Ashbolt *et al.*, 2001). Instead the use of suitable viruses or bacteriophages as models for pathogenic viruses is recommended.

As regards thermal treatment processes, parvovirus is the gold standard by which regulations are set (EU, 2011). Due to its high thermotolerance it is possible to follow the behaviour of this virus along the whole process. However, it has been argued that parvovirus is a much too conservative model organism compared with most pathogenic viruses of interest. Instead, other viruses more similar to the viruses of interest ought to be used when modelling behaviour of pathogenic viruses (Lund *et al.*, 1996).

Another alternative is the use of bacteriophages, viruses that infect bacterial cells. Bacteriophages have a similar structure, morphology and size to many enteric viruses and are only able to multiply within a host cell (Ottoson, 2005; Moce-Llivina *et al.*, 2003; Gantzer *et al.*, 1998; Havelaar *et al.*, 1991). Bacteriophages do not infect all bacteria, but are host-specific. The host specificity is determined by receptors that are expressed on the bacterial cell surface (Goyal, 1987). Some of these receptors are always present on the cell surface, while others are expressed only during limited periods, *e.g.* the F-pili are only expressed during the bacterial growth phase. Bacteriophages that bind to the receptors that are always present on the cell surface are called somatic phages and are the most frequently used of the bacteriophages. In environmental studies, somatic coliphages *e.g.* ΦX174, F-specific RNA (F-RNA) phages *e.g.* MS2 and phages infecting *Bacteroides* spp. are the most commonly used (Leclerc *et al.*, 2000). These phages can be naturally occurring in the environment, while others such as *S.* Typhimurium phage 28B are not commonly found in the environment. Studying bacteriophages along with human and animal viruses of interest can give a good indication of their relevance as viral models. F-RNA phages have been reported to have a lower thermotolerance than somatic coliphages (Lasobars *et al.*, 1999). However, others have reported similar thermotolerance of somatic and F-RNA phages at 60°C and both types of phages are reported to be more thermotolerance than rotavirus, poliovirus and coxsackievirus (Moce-Llivina *et al.*, 2003). Somatic coliphages and F-RNA bacteriophages could therefore be relevant process indicators for viruses in validation processes.

When using process indicator organisms to predict the inactivation of pathogens, care is needed when interpreting the data and when selecting the model organism (Sidhu *et al.*, 1999; Ugwuanyi *et al.*, 1999; Shuval *et al.*, 1991). Preferably comparative studies should be performed to evaluate the usefulness of the model organisms under certain conditions in the environment. In the case of viral models, not only must the structure of the viral particle be similar, but several other characteristics must be taken into consideration. Viruses of similar size and structure can behave in completely different ways due to *e.g.* aggregation and adhesion in and to the matrix used (Gassilloud & Gantzer, 2005).

3.2 Dose-response assessment

It may be difficult to predict the health outcomes due to variations in the host status and between different microorganisms or strains of the same microorganism. There are several possible health outcomes once the infection has begun, *i.e.* asymptomatic illness, acute and chronic disease and mortality.

The relationship between dose and response can be described by mathematical models based on volunteer studies, animal models or outbreak data (Teunis *et al.*, 2010; Haas *et al.*, 1999). Dose-response models translate the estimated dose received in some consuming event into a probability that the dose will result in a transition between health states *e.g.* from healthy to infected, ill, very ill, dead or from infected to ill, very ill or dead. The second health state, also called end-point, is the response, while the dose is the number of pathogens ingested.

The infectious dose of a microorganism is affected by the strain of the organism and by the age and physical condition of the host. Thus the infectious dose can vary widely (Kothary & Babu, 2001). Even if in theory one single organism has the ability to infect a host, the probability of it doing so is low for organisms such as *Salmonella*. Thus *Salmonella* is said to have a high infectious dose (Kothary & Babu, 2001). In contrast to *Salmonella*, other organisms, such as many gastrointestinal viruses, have a high probability of infecting the host even when ingested in low doses, *i.e.* low infectious dose.

However, one should be aware that there are limitations to dose-response models. It is debatable whether it is possible to extrapolate models from one animal species to another, or to humans. Furthermore, the sample population used in feeding-trials might not be representative of the whole population, *e.g.* not including sensitive populations. Depending on the model of choice, it may also be difficult to extrapolate the infection ratio at low doses based on the higher doses commonly used in feeding-trials (WHO, 2003; Haas *et al.*, 1999).

Sensitive populations

Of special interest in a risk analysis are sensitive populations, *i.e.* groups of individuals in greater risk of contracting serious illness, *e.g.* neonates, elderly, immunocompromised individuals, or pregnant women (Gerba *et al.*, 1996). This is also true in animal populations, *e.g.* high-yielding dairy cows are known to be more sensitive to infections. Today the number of humans belonging to sensitive populations is increasing in numbers, especially immunocompromised individuals (Davy, 2002). There is a risk of the risk assessments failing to take into account the high-risk populations since most dose-response models available is based on feeding trials of healthy young individuals thus underestimating the risk associated with sensitive individuals (Haas *et al.*, 1999; Teunis *et al.*, 1996). For this reason the construction of dose-response models using outbreak data is of great interest. However, it is important to remember that in view of the total population the risk might be overestimated when using outbreak studies (Teunis *et al.*, 2010). Due to limited knowledge, accurate incorporation of the high-risk population groups is a challenge to be met in the risk analysis process.

3.3 Exposure assessment

3.3.1 Survival of pathogens

From the time the pathogens are shed from infected animals or humans, the concentration of the organism usually declines over time in the environment (Nyberg *et al.*, 2010; Dumontet *et al.*, 1999).

Bacteria

In general, bacteria are more sensitive to different environmental factors and treatments than viruses and parasites, with the exception of spore-forming bacteria. Spore-forming bacteria are due to their ability to form endospores highly resistant to many sanitisation treatments, and can persist in soil for decades (Mitscherlich & Marth, 1984). The survival of spore-forming bacteria in biogas-plants has been extensively investigated by Bagge (2009). This thesis will not take spore-forming bacteria into consideration.

Since bacteria do not require a host cell for replication, they might multiply in the environment under favourable conditions (Ceustermans *et al.*, 2007; Gibbs *et al.*, 1997; Skanavis & Yanko, 1994). *Salmonella* spp. has been reported to be able to grow in temperature ranging from 6 to 47°C (Mitscherlich & Marth, 1984). Studies of survival in manure amended soils in lysimeters have shown a rapid initial reduction of the bacteria. However, the bacteria could still be detected in low number after 180 days (Nyberg *et al.*,

2010). Other bacteria, such as VTEC, have been shown to survive for up to 10 weeks in faeces. VTEC have also been shown to produce toxin and multiply in faeces at 22 and 37°C (Wang *et al.*, 1996). When it comes to survival, serotypes capable to cause disease, such as O157, do not differ from commensal *E.coli* (Durso *et al.*, 2004).

Some bacteria, for example *Campylobacter* and *Salmonella*, can enter a viable but non-culturable (VBNC) state (Buswell *et al.*, 1998; Kell *et al.*, 1998; Beumer *et al.*, 1992). Bacteria able to enter a VBNC state can be hard to detect in samples with standard culture methods (Gallay *et al.*, 2006; Jones, 2001). This might further contribute to the great diversity in data available on survival of bacteria in organic waste.

Virus

Viruses are the most common cause of food-borne gastrointestinal infections in humans world-wide (Svensson, 2000). However, the risk of transfer of viruses from animals to humans and *vice versa* is mostly small, since viruses often are host- or tissue-specific (Cliver & Moe, 2004). Viruses are resistant to many different types of treatments and harsh environmental conditions, *e.g.* parvovirus and circovirus are very resistant to heat (Emmoth *et al.*, 2004) and enteroviruses have been shown to survive for several weeks in the environment (Vasickova *et al.*, 2005). Due to the fact that they are smaller than bacteria and parasites, viruses are more easily transported in the environment and can cause contamination of, for example groundwater.

As a result of the wide diversity of viruses *e.g.* enveloped/non-enveloped, genome type and replication strategy, a variation in behaviour is to be expected when exposed to sanitisation treatments. In general, the presence of a protein envelope makes the enveloped viruses more sensitive to thermal treatments compared to non-enveloped viruses (Wichuk & McCartney, 2007).

3.3.2 Parameters of importance for microbial survival

There are a number of parameters that affect the survival of microorganisms, such as temperature, pH, moisture content and nutrient availability, all of which are discussed further below. Other factors of importance are oxygen availability, particle size and permeability, presence of organic and inorganic chemical compounds. For example, ammonia (NH_3) chemically hydrolysed or produced by bacteria can inactivate other organisms. To devise proper treatment methods, it is important to have a broad understanding of these factors and their effect on the behaviour of microorganisms.

Temperature

Most pathogenic microorganisms survive well at low temperatures and rapidly die off at high temperatures (>50°C). To ensure inactivation in *e.g*. composting processes, temperatures of around 55-65°C are needed to kill all types of pathogens (except bacterial spores and prions) within hours (Haug, 1993). At lower temperatures, longer retention times during treatment are needed (Sahlström, 2003; Tiquia *et al.*, 1998).

pH

Many microorganisms are adapted to a neutral pH and thus highly acidic or alkaline conditions will reduce their survival. The pH also affects the net charge of viruses and thus their ability to adhere to particles in the matrix (Bitton, 1980). Furthermore the pH has been shown to influence aggregation of viruses (Floyd & Sharp, 1979). Both adsorption and aggregation have been shown to increase the survival of viruses (Gassilloud & Gantzer, 2005; Lewis & Gattie, 2002) and might pose a problem in validation of treatments to reach set reduction targets.

Moisture

Moisture has been known to affect the survival of microorganisms. In general, moisture is required for survival of microorganisms and desiccation will decrease the number of pathogens (O'Callaghan *et al.*, 2001). However, in thermal treatments the conductivity increases with increased moisture, and thus also the inactivation (Nayyeri *et al.*, 2009).

Available nutrients and competing microbiota

If nutrients are available and other conditions are favourable, bacteria can multiply in the environment (Lang & Smith, 2008; Ceustermans *et al.*, 2007). However, enteric bacteria adapted to the gastrointestinal tract are not always capable of competing with indigenous organisms for nutrients and space, limiting their ability to reproduce and survive in the environment (Sidhu *et al.*, 2001).

3.3.3 Possible transmission routes

Transmission routes for pathogens to the environment and from the environment to humans and animals can be through *e.g*. water, food or feed as a result of spreading poorly sanitised products on arable land (Figure 4). Outbreaks of infection and illness in humans and animals have been associated with *e.g*. *E. coli* O157:H7 infection from inadequately washed vegetables (SMI, 2009; Söderström *et al.*, 2008; Cieslak *et al.*, 1993), with garden plots

fertilised with contaminated manure (Mukherjee *et al.*, 2006), and with *Salmonella* infection following irrigation of grazing areas (Jack & Hepper, 1969). Contamination of food-stuffs has also been known to occur as a direct result of faecal contamination during processing (Rabsch *et al.*, 2001). In addition, spread of pathogens can occur through vectors, *e.g.* birds, rodents and insects that have been in contact with the contaminated soil or vegetation.

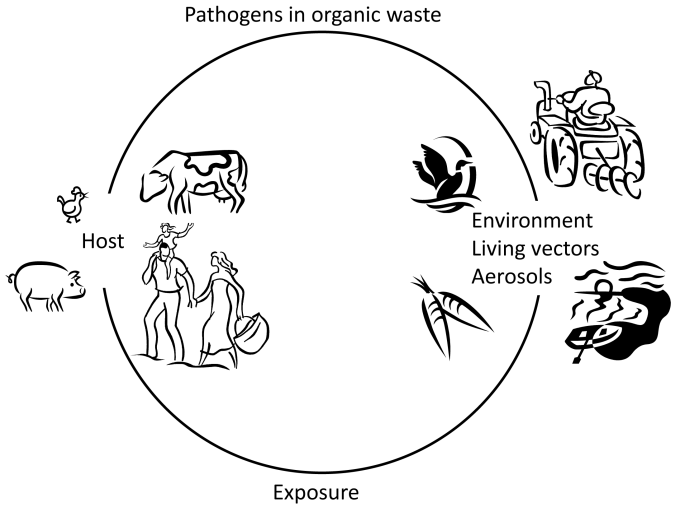

Figure 4. Infection of human and animals can occur as a result of contamination of the environment through recycling of organic waste containing pathogens.

3.3.4 Transmission barriers

The use of barriers in the handling of organic waste is aimed to minimise the risk of transmission of pathogenic organisms in the environment and to humans and animals. Barriers can be applied either before land application, *e.g.* treatment barriers, or after land application.

Treatment barriers

Some of the pathogens found in organic waste have the potential to survive in the material for long periods of time, as mentioned previously. Thus they may end up on agricultural land and on crops if organic waste is used without appropriate sanitation treatment. Sanitation of organic waste can occur through several different kinds of treatment, *i.e.* physical, chemical or biological. Physical treatments include incineration, while ammonia, urea and lime treatment are typical chemical treatments. Biological treatments, composting and anaerobic digestion, can also be used for sanitisation. This thesis does not

consider storage of organic waste, without any form of additional treatment, to be a sanitisation method.

Chemical treatments such as lime addition reduce the microbial content mainly as a result of increased pH, *e.g.* Eriksen et al. (1996) recommend a pH of 12 to inactivate *Ascaris* eggs within 3 months of storage. Ammonia and urea treatments reduce the level of microorganisms when added in sufficient amounts, since ammonia is toxic to microorganisms (Warren, 1962). The efficiency of the treatment has also been shown to be affected by the temperature (Nordin, 2010). The sanitising component during ammonia treatment is un-ionised ammonia (NH_3), while ammonium ions (NH_3^+) are tolerated by most microorganisms (Warren, 1962).

However, this thesis devotes particular attention to sanitation of organic waste through the use of biological and thermal treatment methods. The aim of biological waste treatments is to degrade easily available compounds and stabilise the material. In addition to this, composting reduces the volume of the material (Khalil *et al.*, 2001; Haug, 1993). However, performed under the right circumstances it may also help in reducing the presence of pathogenic microorganisms. During both composting and anaerobic digestion, the most important parameter for achieving a safe end-product has been proven to be the treatment temperature (Ceustermans *et al.*, 2007; Tiquia *et al.*, 1998) (Sahlström, 2003). Composting is a self-heating process during which temperatures lethal to many pathogenic microorganisms can be reached without additional heating (Epstein, 1997). In contrast to the composting process, anaerobic digestion only produces small amounts of heat and the energy is mainly bound in the biogas produced. The anaerobic digestion can run at several different temperatures, from ambient temperatures (psycrophilic and mesophilic) to thermophilic temperatures (>45°C). In small-scale digestion the ambient temperatures are more common. However, inactivation of pathogens through psycrophilic or mesophilic digestion has been shown to be limited (Yen-Phi *et al.*, 2009; Lang & Smith, 2008). Hence, if relying on the process for sanitisation of the substrate, thermophilic digestion, where the materials are heated to high temperatures, is preferable. A separate batch-wise pasteurisation stage, at 70°C for 60 min, following the homogenisation phase for sanitisation of the substrate is today more or less the gold standard for sanitisation treatments if such are applied.

The end-products from composting and anaerobic digestion can be used as fertilisers and soil improvers in commercial soil and compost products. In comparison with compost the digestate have a higher fertiliser value due to a higher content of plant-available nutrients such as nitrogen (Hartmann & Ahring, 2006; Gijzen, 2002).

Efficient sanitisation is reached when the end-product no longer poses a potential health risk to either humans or animals. To devise proper treatment methods, it is important to have an understanding of the effect of different physicochemical parameters on the survival and inactivation of microorganisms and the importance of upholding important parameters throughout the sanitisation treatment of choice. Insufficient time-temperature criteria during thermal treatments or changes in other process parameters, *e.g.* moisture content, might be a cause of pathogens surviving the sanitisation treatment. Non-uniform temperature throughout the whole material due to *e.g.* poor insulation or no insulation at all can also result in pathogen survival.

Reduction targets for sanitisation treatments can be determined through national as well as international regulations and standards. Within the EU, the reduction targets for animal by-products (ABP) category 3 substrates to be used in a composting or biogas plant are set by Commission regulation (EU) No. 142/2011(EU, 2011). The regulation defines sufficient reduction as a 5 $\log_{10}$ reduction in *S.* Senftenberg W775 or *Ent. faecalis* and a 3 $\log_{10}$ reduction in thermotolerant viruses, in the case of thermal treatment options. In the case of chemical treatments, a 3 $\log_{10}$ reduction in the viable stages of resistant parasites, *e.g. Ascaris* sp. should also be shown, in addition to the previously mentioned reduction targets. Certain other requirements are also placed upon the finished end-product, *i.e.* no findings of *Salmonella* in 25g material and on the maximum level of *E. coli* or Enterococcaceae after completion of the sanitisation treatment. Within this thesis, the definition of sufficient reduction follows the reduction targets set by Commission Regulation (EU) No. 142/2011 for thermal treatments unless otherwise stated.

Additional pre- and post-harvest barriers

To further reduce the risk of pathogen transmission associated with recycling of organic waste, additional barriers or restrictions can be applied.

Reduction in pathogens will occur over time in the environment, so leaving a holding time between fertilisation and grazing will eventually reduce the concentration of pathogens in the soil or on vegetation before harvest or grazing. Such restrictions exist in the UK, where a period of two months must elapse between application of soil improvers and fertilisers made from compost from catering waste (*e.g.* household waste) and grazing (Gale, 2002). In the United States two classes, A and B, of end-products are specified. Treated biosolids where pathogens have been reduced to levels that do not pose a significant risk to public health or the environment, but can still be detected, are classified as Class B. For Class B, there are site restrictions preventing access by the public and grazing animals and preventing harvesting of crops

within a specified time. The sanitisation methods used for Class A are assumed to result in a pathogen free product *e.g.* the requirement set for *Salmonella* is a reduction to <3 MPN g^{-1}. The Class A products can be spread without any restrictions (USEPA, 1994). A similar classification was suggested in 2010 for sewage sludge in Sweden by the Swedish Environmental Protection Agency. However, the work with the Swedish regulations is still ongoing and thus no final decisions on classifications or reduction targets for sewage sludge in Sweden have been made (Naturvårdsverket, 2010).

Other barriers can comprise processing of food or feed, *e.g.* washing vegetables and pasteurising milk. Restricting the end-use of fertiliser products to limit the spread of organic waste in fields used to grow food not further processed *e.g.* leafy greens. Nutritional strategies have also been indicated to be of use to decrease the prevalence of *Salmonella* on farm level (Berge & Wierup, 2012). In addition, the choice of application method of the fertiliser can help to reduce the risk of animals coming into contact with the product.

4 Comments on Materials and Methods

In order to clarify the materials and methods used in the Papers I-IV of this thesis, a brief presentation is given here. Additional details are presented in the Materials and Methods section of the individual papers.

4.1 Pathogens and their model organisms

The bacterial pathogens used in Papers I and III were *S.* Senftenberg W775 (NCTC 9959) and *S.* Typhimurium, phage type 178, isolated from Swedish sewage sludge (Sahlström *et al.*, 2004). As models for bacterial pathogens, *Ent. faecalis* (ATCC 29212) and *E. coli* (ATCC 35218) together with *Enterococcus* spp. and total coliforms originating from faecal material or organic waste were studied.

Viral pathogens studied in Papers I and II were highly pathogenic avian influenza virus (HPAIV) strain A/turkey/Italy/1387/00(H7N1) and porcine parvovirus (PPV) strain 893/6 (originally isolated at the Danish Institute for Food and Veterinary Research, Lindholm, Denmark). As models for viruses, the bacteriophages somatic coliphage ΦX174 (ATCC 13706-B1), enterobacteria phage MS2 (ATCC 15597-B1) and *Pseudomonas* phage Φ6 (ATCC 21781-B1) were studied.

4.2 Experimental set-up

4.2.1 Validation of thermal treatment (Paper I)

In Paper I, inactivation of pathogens in dairy cow faeces was investigated using faecal material collected from a dairy herd housed indoors at Kungsängens Research Station, SLU, Uppsala (Figure 5). The material was collected directly from the cows and analysed for pH, dry matter content, volatile solids and C/N ratio. In addition, saline solution was used as a second material to investigate the inactivation in a material with approximately 100% moisture content.

Samples of saline solution and faecal material were inoculated with *S.* Senftenberg W775, *Ent. faecalis*, bacteriophage ΦX174 and PPV. Inoculated samples were mixed manually, weighed into tubes and heat-treated in a preheated block heater, GRANT QBD (Grant Instruments Ltd., Cambridgeshire, UK). Incubation trials were performed at 46.0, 47.5, 49.0, 50.5, 52.0, 53.5 and 55.0°C in saline solution and in cattle faeces. In addition, thermal treatment was performed at 70°C in saline solution. Samples inoculated with bacteria were collected throughout 24 hours of thermal treatment in the temperature interval 46-49°C and 8 hours in the interval 50.5-55°C, while samples inoculated with bacteriophage or PPV were collected throughout 24h of thermal treatment in the interval 46-55°C. At 70°C samples were collected throughout 60 min for *S.* Senftenberg W775, *Ent.* faecalis and the ΦX174 phage and throughout 120 min for PPV.

Statistical analyses were performed using Minitab 15 (Minitab Ltd., Coventry, UK.). Regression analysis using the data obtained from the thermal inactivation studies was performed to obtain the slope, k, of the regression line. D-values were derived from the regression functions when the inactivation throughout the trial period exceeded 1 $\log_{10}$ and at least three sampling occasions were situated within the straight proportion of the inactivation curves. In addition, relationship between D-values and temperature was calculated for the present thermal exposure assessment by regression analysis of the mean $\log_{10}$ D-values versus temperature of the D-values. This made it possible to estimate the reduction in the organisms at any other temperature based on the linear regression model by fitting a regression line to the thermal death time curve between $\log_{10}$ D-values and the corresponding temperature. The straight-line equation obtained was used to calculate a *z*-value, *i.e.* the temperature change required to bring about a 90% change in the D-value.

4.2.2 Composting for AIV elimination (Paper II)

In Paper II, inactivation of H7N1 HPAIV and of bacteriophages MS2 and Φ6 was studied in a compost mixture consisting of poultry manure mixed with straw, with or without the addition of non-hatched eggs. The study was performed both at set temperatures, 35, 45 and 55°C, and in laboratory-scale reactors.

At set temperatures, inoculated samples were mixed manually, weighed into tubes and heat-treated in a preheated block heater, GRANT QBD (Grant Instruments Ltd., Cambridgeshire, UK) or a water bath (Grant OLS200). The pH of the compost mixtures was measured in 1:10 compost mixture/deionised water after incubation for 1 h at room temperature.

Figure 5. Collection of dairy cow manure at Kungsängens Research Station, SLU, Uppsala (left, Paper I), manure-compost at Rölunda Composting Plant, Bålsta (middle, Paper III) and windrow composts at Hovgårdens municipal waste treatment plant, Uppsala (right, Paper III).

Laboratory-scale composting trials were performed in two replicates for each mixture and organism. The first samples (day 0) were collected from the inoculated compost mixtures before the mixtures were packed into laboratory-scale composting reactors (1.5-L Dewar flasks; Fisher Scientific, UK) with Styrofoam lids placed loosely on top of the reactors. Temperature within the reactors was monitored throughout the trial using a TinyTag logger (Intab Interface-Teknik AB, Sweden). Enumeration of viruses and measurement of the pH was performed on days 0, 1, 2, 3 and 7. In addition, moisture content and C/N ratio were determined at the start of the trial (day 0) for all reactors and at the end (day 7) for all reactors with the exception of those inoculated with HPAIV due to biosafety considerations.

Using Minitab 15 (Minitab Ltd, Coventry, UK), linear regression based on the thermal treatment data obtained at set temperatures was performed to determine inactivation rate, expressed as the D-value for each organism and mixture. The D-value was used to extrapolate a 12-$\log_{10}$ reduction (corresponding to over-kill sterilisation) based on the upper 99% confidence interval.

4.2.3 Growth and inactivation in psychrophilic/mesophilic zones (Paper III)

In Paper III, the collected compost materials (Figure 5) were inoculated with microorganisms in order to study the possible growth or inactivation in psychrophilic/mesophilic (P/M) zones of the compost. In brief, fresh cow faeces and material from two municipal household waste composting plants and one manure composting plant were collected and analysed for maturity (Rottegrad and Solvita®), pH, moisture content (MC) and volatile solids.

Inoculated samples were mixed manually and incubated at 14, 24 or 37°C. Following incubation, samples were analysed quantitatively at the start of incubation (day 0) and thereafter during 8 days of incubation.

SigmaStat 3.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses. Bacterial count at the start of the trial was compared with that after 8 days of incubation by Student´s t-test to determine significant inactivation or regrowth. Pearson Product Moment Correlation was used to analyse possible correlations between $\log_{10}$ changes in numbers of *Salmonella* and model organisms, moisture content, volatile solids and pH. Correlations between bacterial count and temperature, Solvita® index and Rottegrad index were analysed using Spearman Rank correlation

4.3 Measurement of maturity

Compost stability is recognised as being an important characteristic of compost for use as a soil conditioner. Many tests are available to determine the stability of the compost, but there is no agreement on which approach is best. In Paper III, the maturity of the compost samples was measured using two methods, Rottegrad and Solvita®.

Rottegrad is a kind of self-heating test, *i.e*. the oxygen uptake is linked to the potential for heat generation. In brief, the moisture content of the compost samples was set using the squeeze test[1]. Samples of approximately 0.8 L were loaded into Dewar flasks and a temperature probe was inserted into the middle of the sample. The Dewar flask was then closed and temperature was monitored as long as heating of the compost occurred. Thereafter the highest temperature recorded was used to determine the maturity on the Rottegrad scale of I-V[2]

Measurement of maturity with the Solvita® kit was performed according to the manual for the test. In brief, samples were loaded into the Solvita® jars up to the fill line and were allowed to equilibrate at room temperature for one hour prior to starting the test. After one hour, CO_2 and NH_3 test gel-paddles were inserted into the jars and the lids were closed tightly. During the test the gel on the paddle did not come into contact with the sample. After 4 hours of incubation at room temperature, the observed gel colour was matched with the colour on the chart supplied with the kit to determine Solvita® CO_2 and NH_3 kit values. The CO_2 value is determined on a scale of 1-8 and the NH_3 value on a

[1] Squeeze test: Take a fistful of compost and squeeze hard. Moisture should appear between the fingers but not drip out if the compost is at the proper moisture content.

[2] Rottegrad scale: I 60-70°C; II 50-60°C; III 40-50°C; IV 30-40 °C; V 20-30°.

scale of 1-5. Thereafter the two values are combined to determine the Solvita® maturity index on a scale of 1-8, 1 being "raw" compost and 8 being "mature/finished" compost.

4.4 Microbial reduction kinetics, D- and z-values

Microbial reduction and regrowth was determined by analysis of viable organisms in the samples throughout the trials.

By plotting the logarithm of surviving cells, N_t, against the time, t, of treatment at temperature T, the following linear relationship is expected to occur:

$$\mathrm{Log_{10}N_t} = \mathrm{Log_{10}N_0} * (\mathrm{k}) * \mathrm{t} \qquad \text{(Equation 1)}$$

where N_0 is the concentration of the untreated population and k is the inactivation rate constant or regression coefficient. In the exponential form, Equation 1 is written as:

$$\mathrm{N_t} = \mathrm{N_0} 10^{kt} \qquad \text{(Equation 2)}$$

Both bacteria and viruses are assumed to follow this first-order exponential decay function. Thus, Equations 1-2 were used to determine the reduction kinetics in Papers I and II. The D-value, *i.e.* the time required to obtain a 1 $\log_{10}$ reduction, is easily obtained using the regression coefficient k' obtained from plotting surviving bacteria or viruses against treatment time:

$$\mathrm{D_{value}} = \frac{1}{-\mathrm{k}} \qquad \text{(Equation 3)}$$

When the detection limit was reached already between the first and second sampling the inactivation rate was calculated using the value of the detection limit and expressed as a maximal D-value.

Biphasic inactivation curves, *i.e.* a high inactivation rate followed by a lower inactivation rate, were observed for enterococci and bacteriophage ΦX174 in cattle faeces (Paper I). Enterocci analysis (PhenePlate analysis) of the flora still remaining at the time corresponding to the second part of the biphasic curve showed no presence of *Enterococcus faecalis*, the strain used to inoculate the samples. Thus the latter part of the inactivation curve was

assumed to correspond to the inactivation rate of naturally occurring microbiota in the cattle faeces. In the case of bacteriophage ΦX174, the fact that adsorption of viruses to solids has been demonstrated to occur resulted in the assumption that the primary part of the curve, was the result of adsorption (Gassilloud & Gantzer, 2005) and thus the second part of the curve was used to calculate reduction kinetics for bacteriophage ΦX174.

The z-value corresponds to the temperature change required to bring about a 90% change in the D-value. Hence, if the z-value = 10°C and the D-value at 70°C has been determined to be 1 minute, then the D-value at 60°C = 10 minutes and at 80°C = 0.1 minutes. The z-values were obtained by linear regression of the plots of $\log_{10}$ D-values against temperature using Equation 4, where k is the slope of the line.

$$z_{value} = \frac{1}{-\mathrm{k}} \qquad \text{(Equation 4)}$$

4.5 Phene Plate analysis

Bacterial isolates of colonies found during analysis of enterococci were sent for subtyping to PhPlate Microplate Techniques AB, Stockholm, Sweden to be analysed using the PhenePlate™-system. PhenePlate analysis is a biochemical fingerprinting method based on 24 different reagents (PhP-FS microtitre plate) (Kuhn *et al.*, 1995). All isolates were compared against reference isolates of *Enterococcus* spp. to obtain a similarity index.

4.6 Collection of data and scenarios for risk assessment

Data for the risk assessment in paper IV were collected through a literature search. When possible, data collected within the research group were used. Furthermore, trials performed under Swedish condition or collected in Sweden were preferred to data from elsewhere. In addition data including presentation of variability were preferred to data presented only as mean or estimated values. The QMRA approach in the present study was to compare mitigation strategies in terms of exposure in grazing cattle after application of organic fertilisers to pastureland, with a total of 80 scenarios (Figure 6).

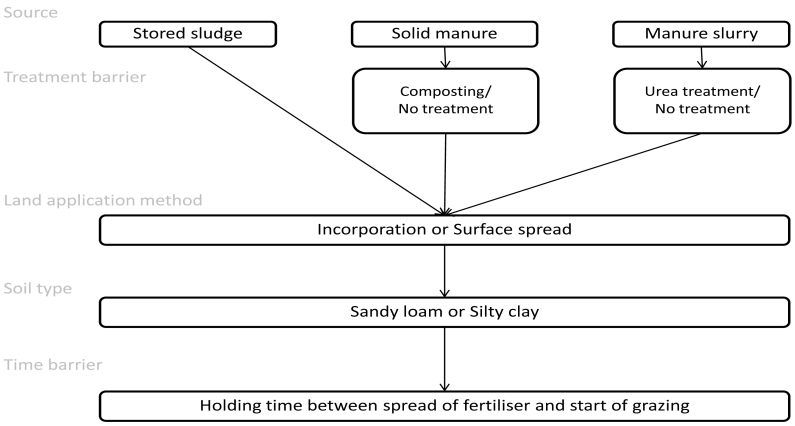

Figure 6. Scenarios included in the risk assessment.

5 Results and discussion - Effect of temperature on inactivation and regrowth of microorganisms

Reduction targets for sanitisation treatments aim to secure a safe end-product for land application, *i.e.* an end-product that poses a minimal risk of pathogen transmission to humans and animals. Regulations for land application of sewage sludge are currently under development in Sweden (Naturvårdsverket, 2010). Thus, it is still uncertain which reduction targets will be set for reuse of sewage sludge. Manure is typically not treated before land application if not used as substrate in a composting plant or transformed into biogas. If used as substrate in a composting or biogas plant ABP are regulated by Regulation (EC) No. 1069/2009 and Commission Regulation (EU) No. 142/2011 (EU, 2011; EU, 2009).

In general, in this thesis a sufficient reduction was assumed to correspond to the targets set by the Commission Regulation (EU) No. 142/2011 for alternative thermal treatment of ABP category 3, which are a 5 $\log_{10}$ reduction in *S.* Senftenberg W775 or *Ent. faecalis* and a 3 $\log_{10}$ reduction in thermotolerant viruses, such as parvovirus, when viruses have been identified as a relevant hazard (EU, 2011). To add a safety margin, treatment times are calculated on the upper limit of the 95% confidence intervals calculated for each D-value. However, under special circumstances a higher reduction target and additional safety margins may apply, for example, when handling materials contaminated with AIV.

5.1 Thermophilic temperatures

Pasteurization for 60 min at 70°C has been the gold standard for thermal sanitisation treatment of organic waste to date. This treatment option is commonly recognized as a method that will produce a safe end-product for land application in terms of minimising the pathogen content. It is approved for

Table 2. *D-values for Salmonella Senftenberg W775, Enterococcus spp, phage ΦX174 and PPV in saline solution and faecal material from cows at temperatures ranging from 46-55°C.*

Treatment temperature (°C)	Saline solution		Faecal material	
	D-value (h)	$CI_{95\%}$	D-value (h)	$CI_{95\%}$
S. Senftenberg W775				
46.0	n.a.	-	22.52	17.1-32.8
47.5	5.37	4.91-5.92	6.23	5.57-7.07
49.0	1.83	1.55-2.24	2.98	2.62-3.44
50.5	0.52	0.45-0.63	1.78	1.60-2.02
52.0	0.48	0.40-0.61	0.82	0.73-0.94
53.5	0.23	0.19-0.27	0.45	0.45-0.41
55.0	0.18	0.14-0.22	0.37	0.31-0.45
Enterococcus spp.*				
46.0	n.a.	-	n.a.	-
47.5	7.58	6.77-8.61	14.5	12.1-18.0
49.0	4.12	3.67-4.71	4.73	4.14-5.53
50.5	1.94	1.94-1.32	1.99	1.74-2.33
52.0	1.44	1.44-1.31	0.89	0.72-1.17
53.5	0.57	0.57-0.46	0.71	0.53-1.06
55.0	0.39	0.39-0.35	0.45	0.34-0.66
ΦX174				
46.0	25.7	19.6-37.2	16.5	12.6-23.4
47.5	20.6	18.3-23.7	17.4	13.8-23.4
49.0	12.1	10.8-13.7	16.9	16.9-19.9
50.5	7.23	6.40-8.31	3.43	2.73-4.62
52.0	4.63	3.97-5.57	3.13	2.53-4.10
53.5	1.64	1.41-1.95	1.69	1.46-2.00
55.0	1.35	1.20-1.54	1.51	1.24-1.93
PPV				
49.0	n.a.	-	17.0	12.9-24.8
52.0	n.a.	-	16.8	13.8-21.3
55.0	22.3	16.9-32.6	10.8	8.85-14.0

* Only *Enterococcus faecalis* in the case of saline solution; n.a., not applicable due to <1 log10 reduction detected during the trial period.

processing of ABP in accordance with the Commission Regulation (EU) No. 142/2011 (EU, 2011). At such a high temperature, the survival time of most pathogens is short (Sahlström *et al.*, 2008; Jones & Martin, 2003). The results presented in Paper I show pasteurization at 70°C in saline solution to be

effective to reduce *S.* Senftenberg W775 and *Ent. faecalis*. Both bacteria were reduced to below the detection limit within 5 min of pasteurization, corresponding to a reduction of >6.5 and >5.4 $\log_{10}$ in *S.* Senftenberg W775 and *Ent. faecalis* respectively.

The D-value of 1.2 h ($CI_{95\%}$ 1.05-1.51 h) for PPV, calculated from the data obtained in Paper I, results in a required retention time of approximately 4.5 h at 70°C to obtain a 3 $\log_{10}$ reduction, based on the upper limit of the 95% confidence interval. While sufficient reduction of the ΦX174 phage, suggested by Bertrand *et al.* (2012) to be relevant viral model organism, was reached well within 60 min (with a D-value of 1.8 min, $CI_{95\%}$ 1.6-2.1).

However, heating substrates to temperature as high as 70°C is expensive and the process also needs to be performed in a separate batch process. Thus, for economic and practical reasons, validation of alternative treatments is of interest.

The results (Paper I) suggest that day-to-day sanitisation treatments ought to be performed at temperatures above 50°C in order to reach sufficient reduction targets and produce a safe end-product within a reasonable time (Table 2). While lower temperatures would eventually reduce the pathogens to a sufficient level, this would lead to prolonged survival and increased uncertainty in the inactivation rate due to higher variation in inactivation rates at lower temperatures (Table 2). In some cases it would also result in an unreasonably long retention time for the sanitisation treatment, *e.g.* 164h at 46°C to reach the reduction target for *S.* Senftenberg W775 based on the upper limit of the 95% confidence interval.

In the case of thermal treatment of *Ent. faecalis* and the ΦX174 phage, in cattle faeces, biphasic inactivation curves were observed. This phenomenon has previously been described for viruses and is assumed to be the result of adsorption of the virus to solids in the sample (Gassilloud & Gantzer, 2005), which was also assumed to be the case in this study. In the case of enterococci, a change in the bacterial flora could be seen in the shift between the first and second slope of the inactivation curve. Analysis of the bacterial flora remaining in the later stages of the thermal treatments showed the presence of *Ent. faecium* among other unidentified *Enterococcus* spp.. Further examinations of the inactivation rates of the flora remaining during the later stages of the inactivation curve showed a higher thermotolerance of this subpopulation, 0.53 h at 55°C in saline solution compared with 0.39 h determined for *Ent. faecalis* at 55C in saline solution. Hence, for calculation of inactivation rates for enterococci the first part of the slope was used, assuming that the second part correspond mainly to other *Ent.* spp. Since there is a risk of

over- or underestimating the inactivation rate, great care has to be taken when deciding upon which of the two slopes used for calculation of the D-value.

Thermal treatment of PPV at 49, 52 and 55°C showed a high thermotolerance in the virus. In saline solution the inactivation was in fact less than 1 $\log_{10}$ over 24 h at 49 and 52°C, while in faecal material a higher inactivation rate could be seen. However, it remains unclear if the difference in inactivation rates in between the two materials is true inactivation or a result of adsorption to solids in the faecal material.

Enveloped viruses, for instance AIV, are known to be more sensitive to thermal treatment then non-enveloped viruses (Wichuk & McCartney, 2007). Hence it is not surprising to see that thermal treatment at 55°C is sufficient to rapidly inactivate highly pathogenic avian influenza virus (HPAIV) in poultry manure compost, irrespective of material composition, with a D-value of 2.4-2.5 min.

5.2 Mesophilic temperatures

Previous studies have shown that thermal treatment under mesophilic conditions is insufficient in reducing pathogens such as *Salmonella* spp. and *E. coli* O157:H7 (Yen-Phi *et al.*, 2009; Lang & Smith, 2008; Lund *et al.*, 1996; Kearney *et al.*, 1993). Furthermore, low temperatures (psycrophilic and mesophilic) under favourable conditions can result in regrowth of bacterial pathogens, as shown in Paper III and supported by several other published studies (see section 5.3).

However, depending on the heat sensitivity of the organism of interest, lower treatment temperatures might be sufficient to reach the reduction targets. Mesophilic temperatures, 35 and 45°C, were sufficient to reach a high reduction in the enveloped HPAIV in the study of thermal inactivation in poultry manure compost (Paper II). Similar results were found for the enveloped Φ6 phage. Comparing these results to the more thermotolerant bacteriophage MS2 showed a great difference in heat-resistance. At 35°C the MS2 phage was not reduced with even 1 $\log_{10}$ during the study period and at 45°C the experiments resulted in a D-value of >2.6 days.

The results obtained at set temperature were confirmed in the complementary study using laboratory-scale composting of poultry manure to eliminate HPAIV and the phages Φ6 and MS2 (Paper II). Composting was shown to rapidly reduce the infective titre of HPAIV and the Φ6 phage (Table 3). Even when the compost did not reach thermophilic temperatures, HPAIV was effectively reduced to below the detection limit within 24 h from the start of the trial.

Table 3. *Concentration of HPAIV (log_{10} $TCID_{50}$/g), Φ6 and MS2 phage (log_{10} PFU/g) during laboratory-scale composting (day 1 and 8) of poultry manure (mixture 1) and with addition of 25% non-hatched eggs by weight (mixture 2) and time required to reach a 12 log_{10} reduction at a set temperature of 35°C based on the upper 95% confidence interval limit of the D-value.*

Organism	Mixture 1				Mixture 2			
	Day 0	Day 7	Mean reduction	12 log_{10}	Day 0	Day 7	Mean reduction	12 log_{10}
HPAIV	4.7±0.2	<1.8	>2.9	6.4 h	5.1±0.2	<1.8	>3.3	7.6 h
Φ6	7.5±0.1	<1.0	>6.5	5.4 h	7.1±0.2	<1.0	>6.1	5.3 h
MS2	7.7±0.1	3.4±0.3	4.3	n.i.	7.5±0.0	<1.0	>6.5	n.i.

5.3 Bacterial regrowth potential

In contrast to viruses, bacteria have the ability to multiply outside the host. Thus, under favourable conditions in the environment or during insufficient sanitisation treatments, they may increase in amount instead of decreasing (Ceustermans *et al.*, 2007; Gibbs *et al.*, 1997; Skanavis & Yanko, 1994).

Table 4. *Maturity (Rottegrad and Solvita®) of substrate from household waste compost, beef cattle manure compost and dairy cattle faeces. Results of comparison of the bacterial count after 8 days of incubation with bacterial counts of S. Typhimurium and Enterococcus spp. at the start of the study. Significant results of student's t-test given as *(p<0.05) ** (p<0.01) and *** (p<0.001), growth of microorganisms indicated in bold.*

Sample no.	Maturity		*S.* Typhimurium			*Enterococcus* spp.		
	Rottegrad	Solvita®	14°C	24°C	37°C	14°C	24°C	37°C
Household waste compost								
A1	III	4	**1.1****	**1.8*****	-1.9**	**2.3*****	**1.2***	-1.6*
A2	IV	4	**0.5**	**1.4****	**0.9****	**1.8*****	**1.4****	-0.4
A3	III	5	-1.0*	-1.4**	-1.9***	-0.2	-1.6	-3.0***
B1	IV	6	**0.7**	**1.1***	**0.4**	**1.7****	**1.9***	-0.3
B2	IV	8	-3.2**	-3.4	-4.3***	-2.2	-2.0	-3.0**
B3	II	3	-0.7*	-0.8**	-1.5	**0.4**	**0.3**	-0.5*
Beef cattle manure compost								
C1	IV	5	-1.4***	-2.1***	-2.4***	**0.4**	-0.3	-1.1
C2	V	7	-0.5***	-0.8***	-3.0**	**0.8*****	0.5**	-1.0
C3	V	7	-0.4**	-0.9**	-2.2**	**0.8*****	**0.2**	-0.7
C4	V	8	-0.5*	-0.8**	-2.8***	-0.5	-0.6**	-0.8
C5	V	8	-1.1*	-1.4**	-2.6***	-0.6*	-1.0**	-1.3**
C6	V	8	-1.2***	-1.7***	-2.4***	-0.4*	-0.6*	-0.5
Fresh dairy cattle faeces								
D1	-	4	**0.3**	**0.5***	-0.8	-0.1	-0.6	-2.0*

Investigation of regrowth potential in compost material in Paper III revealed that growth of *S.* Typhimurium and coliforms can occur in substrate from household waste compost of low maturity and in dairy cattle faeces at mesophilic and psycrophilic temperatures (Table 4). Similarly to *Salmonella, Enterococcus* spp. was found to be able to regrow in substrate from household waste compost and in substrate from beef cattle manure compost but not in dairy cattle faeces (Table 4). Thus, unless a uniform high temperature is maintained throughout the whole material, there is a risk that the treatment will not live up to the required reduction targets. This topic is further discussed in section 7.2. In general, there seems to be an increased risk of regrowth of bacterial pathogens in materials of low maturity, while no regrowth occurs in mature materials, possibly due to lack of the nutrients needed for bacterial growth. Thus, the risk of regrowth in the finished and mature end-product ought to be limited.

5.4 Time- and temperature-dependent inactivation models

The results from Paper I show that microbial inactivation rates are related to the temperature and duration of thermal treatment. To study the inactivation rate for the organisms included in Paper I, regression models were based on the input variables time and temperature in cattle faeces and saline solution to obtain D-values (Table 2). For each of the organisms studied, the $\log_{10}$ of the D-values was plotted against temperature and a regression was fitted. Z-values and their 95% confidence interval, presented in Table 5, were derived from the regression function (reciprocal of the slope of the regression) of the $\log_{10}$ D-values and the temperature. Z-values for *S.* Senftenberg W775 were found to be similar in between the two matrices, saline solution and faeces, while the z-value for enterococci was higher in saline solution compared to faeces.

The trend of increased sensitivity to temperature in saline solution compared with faecal material seen for the ΦX174 phage in our studies of z-values supports findings by Bertrand *et al.* (2012). Those authors presents an extensive study of viral inactivation in simple and complex matrices, based on a meta-analysis of literature data on the time to the first log reduction. The results show a lower sensitivity to temperature change in complex matrix at temperatures >50°C while the sensitivity to changes at lower temperatures is similar, irrespective of matrix.

Table 5. *Regression equations based on data on microbial inactivation and z-values (°C) from Paper I and the 95% confidence intervals (CI) in saline solution and cattle faeces.*

Organism	Saline solution		Cattle faeces	
	Regression equation	z-value (95% CI)	Regression equation	z-value (95% CI)
S. Senftenberg W775	y = -0.1938x+9.7649	5.16 (5.09-5.23)	y = -0.1951x+10.139	5.13 (5.09-5.17)
Ent. spp.	y = -0.1748x+9.1722	5.72 (5.70-5.75)	y = -0.1976+10.39	5.06 (5.00-5.12)
ΦX174	y = -0.1537x+8.5709	6.51 (6.48-6.54)	y = -0.1397x+7.7858	7.16 (7.10-7.21)
PPV		n.d.	y = -0.0325x+2.854	30.76 (30.68-30.84)

* Only *Ent. faecalis* in saline solution; n.d. not determined due to lack of data.

A broad span of D-values obtained for the same organism in different studies on bacterial and viral inactivation has been presented in the literature (Bertrand *et al.*, 2012; Lang & Smith, 2008; Ceustermans *et al.*, 2007; Soldierer & Strauch, 1991). Even if the most important parameter during thermal treatment is thought to be the treatment temperature (Ceustermans *et al.*, 2007; Sahlström, 2003; Tiquia *et al.*, 1998) other parameters also affect the process efficiency. Some of the differences between studies can be explained by differences in the matrix used in the trials, for instance the moisture content (MC) of the materials may play an important role. Increased MC can be linked to decreased D-value, probably due to increased conductivity in the materials at high MC. This can be seen on comparing the D-values in studies with materials of different dry matter content, *e.g.* D-values at 55°C for *S.* Senftenberg W775 of 11 min at MC approximately 100%, 22 min at MC 13.4% (Paper I), and 104 min at MC 60% (Ceustermans *et al.*, 2007). Turner and Burton (1997), on the other hand, suggest that solids within the material can lead to increased heat retention in the material, thus leading to increased inactivation of microorganisms.

In an attempt to evaluate the z-value obtained for *S.* Senftenberg W775 in faecal material from dairy cows, additional data on inactivation rates were collected from the literature. *Salmonella* spp. is a frequently studied group of pathogens and several studies have presented D-values in a variety of materials that might end up as organic waste, *e.g.* manure and meats (Wagner *et al.*, 2009; Lang & Smith, 2008; Ceustermans *et al.*, 2007; McCormick *et al.*, 2003;

Juneja *et al.*, 2001; Blackburn *et al.*, 1997; Soldierer & Strauch, 1991). Summing up data from Paper I and literature data allows for a broader estimation of inactivation rates in organic waste at thermophilic temperatures (Figure 7). This strategy resulted in a change in the slope and increased the z-value for Salmonella by 1.7°C, from 5.1 to 6.9°C. It is probable that the change in z-value is a combined result of differences in heat sensitivity between *Salmonella* strains, but also a matrix effect, for instance, differences in MC. However, as for viral inactivation (Bertrand *et al.*, 2012), the literature contains a limited amount of studies on the survival and inactivation of *Salmonella* in the interface of mesophilic and thermophilic temperatures. Thus, the data added to the model in Figure 7 span from 50-70°C, leaving the lower interval of thermophilic temperatures with a small amount of data. To further refine the model for thermal treatment of organic waste at thermophilic temperatures, further literature searches for data, especially in the lower end of the range, would be of interest. Due to the change in z-values seen when adding additional data from other matrices it might be desirable to develop the model further.

5.5 Model organisms and process indicators

With today's great advances in detection of organisms using molecular tools, *e.g.* different PCR techniques, it might be argued that the use of model organisms and indicators is reduced. Nevertheless, in validation of sanitisation treatments process indicators are useful, since it would be far too costly to analyse all pathogens of interest present in the material. Furthermore, as most techniques do not only recognise viable organisms, setting treatment recommendations based on data from such studies risks underestimating the treatment efficiency.

By using indicator organisms, certain generalisations are allowed to be made and validation of methods can be based on the assumption that the process indicator chosen presents a kind of a worst-case scenario. Thus one important feature for an model organism is that it should be slightly more resistant to the treatment in question than the pathogen (Haug, 1993). For example, the use of *S.* Senftenberg W775 in validation of treatments is based on the assumption that this particular strain possesses a higher thermotolerance than other *Salmonella* spp. (Henry *et al.*, 1969). Parvovirus is also used as a process indicator for viral reduction in validation of alternative processes, as previously mentioned. A summary of laboratory setups throughout Papers I-III is presented in Table 6.

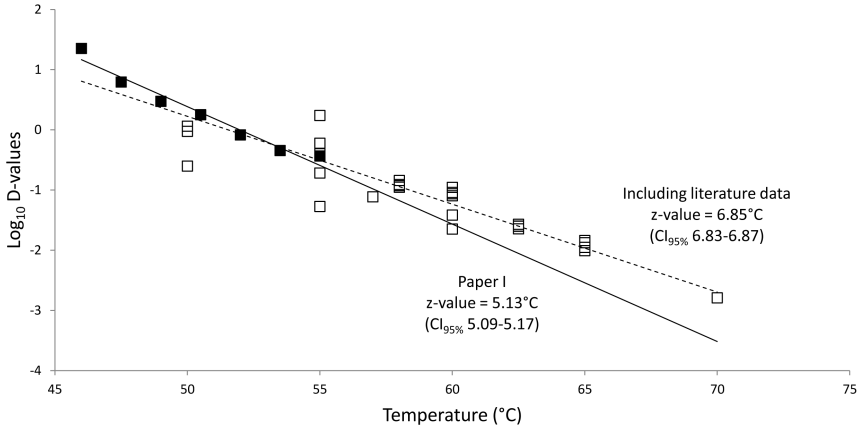

Figure 7. Plot of $\log_{10}$ of the D-values (h) in Paper I (filled squares) and literature data (open squares) for *Salmonella* Senftenberg W775 and *Salmonella* spp., respectively, and z-values (°C) including the 95% confidence interval.

5.5.1 Bacterial models

In this thesis, enterococci (Papers I and III) and total coliforms (Paper III) were investigated as models for *Salmonella* during inactivation and regrowth in faecal matter and organic waste. Enterococci and total coliforms might be useful as process indicators because they are already present in materials such as organic waste in sufficient amounts. In Paper III, the regrowth patterns of *S.* Typhimurium and enterococci were shown to be similar. During inactivation trials, previous studies have shown enterococci to possess a higher thermotolerance than Salmonella (Vinnerås, 2007; Craven *et al.*, 1997; Shuval *et al.*, 1991) and thus to be a conservative process indicator. In Paper I, inactivation rate of enterococci during thermal treatment of faecal matter and saline solution was similar to that of *S.* Senftenberg W775 but in most cases slightly lower, thus supporting the idea that enterococci is a conservative indicator for *Salmonella*. However, assuming that the task is to find a proper process indicator that represents a worst-case scenario, enterococci may be deemed suitable.

However, several of the traditionally used faecal indicators *e.g.* enterococci, *E. coli* and coliforms, have been shown to not be suitable model organisms for pathogens, including some bacteria and most viruses and parasites (Harwood *et al.*, 2005; Ashbolt *et al.*, 2001; Grabow, 2001).

Table 6. *Model organisms used within studies in Paper I-III*

Pathogen	Model organism	Comments	Paper(s)
Salmonella	Enterococci	+ Similar growth and inactivation pattern as *Salmonella*, similar z-value - Slightly more thermotolerant *i.e.* higher D-values	I, III
	E.coli	+ Similar growth pattern	III
PPV	Bacteriophage ΦX174	- Significantly more heat-sensitive.	I
HPAIV	MS2	+ Possible to study throughout the process - Significantly more thermotolerant	II
	Pseudomonas phage Φ6	+ Similar inactivation pattern to HPAIV - More difficult to analyse than other phages, rapid inactivation also at low temperatures	II

5.5.2 Viral models

Parvovirus is an extremely thermotolerant virus (Lund *et al.*, 1996; Bøtner, 1990), in contrast to the vast majority of viruses present in organic waste (Bertrand *et al.*, 2012; Emmoth *et al.*, 2004; Bøtner, 1990). Studying the literature shows a wide spread of D-values of parvovirus, some of them showing a 3 $\log_{10}$ reduction in the virus within the hour and others showing a much lower inactivation rate, *e.g.* a 1.2 $\log_{10}$ reduction (Paper I). Compared with the standard for validation of alternative sanitisation treatments, a 3 $\log_{10}$ reduction in thermotolerant viruses such as parvovirus, it is questionable whether even the standard of pasteurization for 60 min at 70°C meets this reduction target, which further emphasises the extreme thermotolerance of PPV.

Using parvovirus as the choice of viral process indicator might in some way be appropriate. If a reduction target is set for such a thermotolerant virus, it is almost certain that most other known viruses of interest will also be sufficiently reduced. Furthermore, the use of parvovirus might be well motivated in comparative studies when wanting to explore the relative effect of different inactivation treatments, since long survival makes it possible to study the effect of the treatment concerning viral inactivation over extended periods. At the same time, it can be argued that the difference in thermotolerance between the process indicator and most other viruses of interest in this case is too great and that other more relevant viruses should be used as indicators in validation of sanitisation treatments. It has therefore been suggested that when

setting demands on sanitisation treatments, it can be more reasonable to use less heat-resistant viruses, ones that are more similar to other pathogenic animal viruses, *e.g.* reovirus or picorna virus (Lund *et al.*, 1996). Bacteriophages such as ΦX174 phage might also be promising models for viruses in sanitisation treatments, as they have been shown to be more thermotolerant then many other viruses, including Hepatitis A (Bertrand *et al.*, 2012).

The non-enveloped bacteriophage ΦX174 was shown to be considerably less thermotolerant than PPV in the interval of 46-55°C and also at 70°C (Paper I). While the 95% confidence intervals obtained for the D-values of PPV and ΦX174 overlapped in some cases, this was most likely due to the large confidence interval as a result of small or negligible reduction in infective titre of PPV.

In general, the non-enveloped, F-RNA bacteriophage MS2 was the most thermotolerant phage studied in this thesis. Barely any inactivation could be seen at 35, 45 or 55°C (Paper II). The phage proved far too thermotolerant to be a valuable process indicator for HPAIV. However, it might be a suitable indicator for more thermotolerant viruses such as PPV.

Like other enveloped viruses, the Φ6 phage is relatively sensitive to heat and thus it is not surprising that the phage was reduced easily during thermal treatment even at temperatures as low as 35°C (Paper II). In Paper II, as a result of the initially higher titre of the Φ6 phage compared with the HPAIV comparison of calculated D-values may be misleading, resulting in an apparently higher inactivation rate of the phage then the HPAIV. At the same time the possibility of propagating the indicator to higher titres compared with the pathogen and thus being able to study the inactivation throughout a longer period, is a valuable property of a process indicator. In fact, due to rapid inactivation of both the Φ6 phage and HPAIV, no difference in inactivation rates could be detected. Thus the Φ6 phage is a promising candidate as a process indicator for AIV and possibly also other viruses sensitive to thermal treatments. This confirms findings by Adcock *et al.* (2009), who studied the Φ6 phage in comparison with H5N1 HPAIV and concluded that it may be suitable as an indicator organism for AIV.

6 Results and discussion – Risk assessment

6.1 Is there a need for QMRA?

The objectives for performing a QMRA concerning spread of manure and sewage sludge onto arable land and pasture (Paper IV) were:

- The lack of general knowledge of how different barriers affect the risk of transmission of *Salmonella* from fertilisers used on land to grazing cattle;
- To provide decision support for stakeholders, researchers and managers as a basis for risk management and risk communication;
- To provide data that could be used for future comparisons between barriers used to reduce the pathogen level before and/or after land application of manure and sewage sludge;
- To identify relevant knowledge gaps which need to be filled in order to refine the QMRA model.

6.2 Scenarios for exposure

The transmission scenarios examined included fertilisation of land with solid manure, manure slurry or sewage sludge (Figure 6). The amount of soil ingested was assumed to be 0.33 kg for sandy loam and 0.36 kg for silty clay expressed in wet weight, based on consumption of 13.7 kg dry matter per animal of which 2.25% is soil (Thornton & Abrahams, 1983)

The levels of viable *Salmonella* in the three sources were estimated using Swedish data on prevalence when possible. Occurrence of *Salmonella* in sewage sludge stored for six months, which is in accordance with current recommendations for land application of sewage sludge (REVAQ, 2011), was estimated at an expected value of 240 CFU kg^{-1} wet weight (Leander *et al.*, 2012). *Salmonella* prevalence in solid manure and manure slurry was estimated based on level of shedding, within-herd prevalence, between-herd prevalence

and a 180-fold dilution of manure in the manure pit. Furthermore, semi-continuous storage of solid manure and manure slurry was assumed with an inactivation rate of 0.07 and 0.08 $\log_{10}$ day^{-1}, respectively (O´Neill *et al.*, 2011), during 365 days.

Thermal treatment of manure in order to reach a 5 $\log_{10}$ reduction was based on the recommendations for thermal treatment at 50.5°C in Paper I and for urea treatment of manure slurry data presented by Ottoson *et al* (2008) at 14°C, with an addition of 2% urea. By setting the time requirement for each sanitisation treatment based on the upper limit of the 95% confidence interval for the D-value, additional safety margins were obtained.

Application and dilution rates in soil were obtained from recommendations by the Swedish Board of Agriculture. Data on the reduction in soil after land application by incorporation were obtained from Nyberg *et al* (2010) and increase in reduction when the materials are surface-spread, was based on findings by Hutchison *et al* (2004). For a detailed list of distributions and parameters, see Paper IV.

6.3 Exposure assessment

The lack of a dose-response model for cattle meant that the end-point was the exposure dose of viable *Salmonella* during the first day of grazing after fertilisation of the pasture. Comparison of the scenarios showed that sanitisation treatments reduced the exposure dose per 10,000 cattle to below 0 CFU within 6 days of holding time, irrespective of the source material, application method and soil type (Paper IV). When surface-spread, the dose per 10,000 cattle fell to 0 within 30 days of holding time even if the materials had not been sanitised before land application, while incorporation of the materials led to prolonged survival of *Salmonella*. Based on these finding, the worst-case scenario would be to let grazing animals onto pasture, without a holding time between fertilisation and start of grazing, with fertilisation comprising untreated solid manure followed by sewage sludge and untreated manure slurry.

The dose for the exposure scenario which corresponds to the best-case scenario, *i.e.* the scenario resulting in the lowest dose per source and soil, is summarized in Table 7.

Comparing the estimated dose in terms of relative exposure makes the results easier to communicate. The relative increase in dose compared with the best-case scenarios in sandy loam and silty clay soil is presented in Tables 8-9. The addition of both sanitisation steps and a holding time of 30 days was estimated to lower the dose by at least 10^{11}-fold. Comparison of the untreated

Table 7. *Estimated dose (CFU) ingested by cattle on day one of grazing in the best-case scenario, corresponding to surface application of the material, sanitisation treatment, in case of solid manure and manure slurry, and a holding time of 30 days.*

Soil type	Source		
	Sewage sludge	Treated solid manure	Treated manure slurry
Sandy loam	1.4×10^{-6}	7.8×10^{-11}	1.2×10^{-13}
Silty clay	8.5×10^{-8}	4.6×10^{-12}	7.2×10^{-15}

materials showed a 24-25-fold increase when comparing solid manure with sludge and sludge with manure slurry. Land application of solid manure resulted in a 595-598-fold increase in dose compared with manure slurry.

6.4 Comments on data analysis and parameters

The exposure dose in cattle will depend on source, sanitisation treatment and the time that elapses between land application and start of the grazing, *i.e.* the holding time. Sensitivity analysis showed that the variation in the present input parameters for the soil reduction had the largest influence on dose. Other parameters that have a large influence on the dose are variation in the level at source and variation in the effectiveness of the sanitisation treatments.

Risk assessment not only identifies differences between different fertiliser sources and mitigation strategies, but also highlights areas that would benefit from increased knowledge. Some comments on the parameters and distributions used are presented below.

There is currently not sufficient knowledge concerning the prevalence of *Salmonella*, both in manure and sewage sludge, in Sweden. Lack of knowledge of the amounts that are shed from infected animals and within-herd prevalence is reflected by the use of uniform distributions. The uniform distribution is rarely a good way of describing variation, since it gives each value the same probability between the min and the max value, and values falling just outside the limits have a probability of 0. However, in illustrating uncertainty, either uniform or PERT distributions are recommended (Haas *et al.*, 1999). At present, new bulk tank milk screening is prepared and will provide new results, so the data on the prevalence of *Salmonella* in Swedish dairy herds can be updated. Due to the lack of data on the prevalence of *Salmonella* in raw sludge, the level in sludge stored for 6 months has been estimated with a standard deviation of 0.8 (Leander *et al.*, 2012; van Schothorst *et al.*, 2009) based on a recovery of 50% in analysing the samples. However, the data available for this estimation are limited and further sampling is needed to increase the knowledge of *Salmonella* in sewage sludge.

Table 8. *Relative increase in dose for cattle compared with the best-case scenario (Table 7), sandy loam soil*

Source	Land application method	Holding time after application			
		0	6	16	30
Sewage sludge					
	surface	5.4×10^{5}	1.9×10^{4}	360	1
	incorporated	5.4×10^{5}	9.7×10^{4}	1.3×10^{4}	610
Solid manure					
without treatment	surface	2.4×10^{11}	8.7×10^{9}	1.6×10^{8}	4.5×10^{5}
	incorporated	2.4×10^{11}	4.4×10^{10}	5.7×10^{9}	2.8×10^{8}
thermal treatment	surface	5.4×10^{5}	1.9×10^{4}	360	1
	incorporated	5.4×10^{5}	9.7×10^{4}	1.3×10^{4}	610
Manure slurry					
without treatment	surface	2.6×10^{11}	9.3×10^{9}	1.7×10^{8}	4.8×10^{5}
	incorporated	2.6×10^{11}	4.7×10^{10}	6.1×10^{9}	2.9×10^{8}
thermal treatment	surface	5.4×10^{5}	1.9×10^{4}	360	1
	incorporated	5.4×10^{5}	9.7×10^{4}	1.3×10^{4}	610

Table 9. *Relative increase in dose for cattle compared with the best-case scenarios (Table 7), silty clay soil*

Source	Land application method	Holding time after application			
		0	6	16	30
Sewage sludge					
	surface	9.8×10^{6}	$3{,}4\times10^{4}$	187	1
	incorporated	$9{,}8\times10^{6}$	$5{,}3\times10^{5}$	$3{,}7\times10^{4}$	$2{,}5\times10^{3}$
Solid manure					
without treatment	surface	$4{,}4\times10^{12}$	$1{,}5\times10^{10}$	$8{,}5\times10^{7}$	$4{,}5\times10^{5}$
	incorporated	$4{,}4\times10^{12}$	$2{,}4\times10^{11}$	$1{,}7\times10^{10}$	$1{,}1\times10^{9}$
thermal treatment	surface	$9{,}8\times10^{6}$	$3{,}4\times10^{4}$	187	1
	incorporated	$9{,}8\times10^{6}$	$5{,}3\times10^{5}$	$3{,}7\times10^{4}$	$2{,}5\times10^{3}$
Manure slurry					
without treatment	surface	$4{,}7\times10^{12}$	$1{,}6\times10^{10}$	$9{,}1\times10^{7}$	$4{,}8\times10^{5}$
	incorporated	$4{,}7\times10^{12}$	$2{,}6\times10^{11}$	$1{,}8\times10^{10}$	$1{,}2\times10^{9}$
thermal treatment	surface	$9{,}8\times10^{6}$	$3{,}4\times10^{4}$	187	1
	incorporated	$9{,}8\times10^{6}$	$5{,}3\times10^{5}$	$3{,}7\times10^{4}$	$2{,}5\times10^{3}$

Storage of manure has been assumed to be 365 days, in heap or manure pits with a semi-continuous addition of manure containing a constant level of *Salmonella*. In the approach used in this thesis, the reduction was assumed to follow first-order kinetics, without any regrowth. However, during storage a large amount of different environmental parameters can influence the reduction rate (see section 3.3.2). In the case of manure, the fact that the level of *Salmonella* and the storage time might vary greatly also has to be taken into consideration. There is also a risk of growth of *Salmonella* in manure, as illustrated in Paper III.

Inactivation of *Salmonella* during sanitisation treatments, similarly to the inactivation during storage, was assumed to follow first-order kinetics. Furthermore, it was assumed that the recommended treatment times, temperatures and concentrations are maintained throughout the treatment to ensure a reduction of at least 5 $\log_{10}$. It was thus assumed that the stated parameters are those that play the most important role in the survival of *Salmonella* during treatment.

The rates of inactivation in different soils were estimated from published data from lysimeter trials (Nyberg *et al.*, 2010). However, as in the case of storage several environmental parameters can influence the inactivation rate, *e.g.* weather conditions, and the parameter can therefore vary greatly. It is thus probable that the inactivation rates will vary between year and seasons, resulting in a high variability in the parameter in the model.

Following increased knowledge of sources, survival and inactivation and additional collection of data to better illustrate the variation in parameters used in the model, the model should be refined so as to increase the accuracy of the risk estimate.

6.4.1 Sensitive populations

Even though every single bacterium has the potential to cause infection the infectious dose of *Salmonella* is said to be relatively high (Kothary & Babu, 2001). There is also a difference in sensitivity to infection between different groups within a population (Gerba *et al.*, 1996). Thus it can be recommended that extra caution be taken with such groups, *e.g.* high-yielding dairy cows and young calves, while *e.g.* shorter holding times can be applied to pasture for other groups of healthy animals. Determination of the assumed negligible dose is part of the risk management process and has to take into consideration not only the effect of different barriers, but also a cost-benefit analysis to determine whether the reduction in dose is reasonable compared with the increase in cost.

7 Concluding discussion

7.1 The multi-barrier approach to safe recycling

As been shown, sanitisation treatments are effective in rapidly reducing the amount of pathogens before land application of organic waste. Sanitisation treatment of solid manure and manure slurry resulted in a decreased exposure dose of *Salmonella* by at least 10^5, which is reasonable taking into account the assumption of first-order kinetics of inactivation of *Salmonella* during the treatment. The reason for not sanitizing stored sludge is the REVAQ recommendations for 6 months' storage of sewage sludge before application to arable land (REVAQ, 2011). Here it is of great importance to emphasise that storage, is not a sanitisation treatment and even if some reduction occurs during storage it is difficult to foresee the size and reliability of the reduction. Furthermore, it must be remembered that according to current regulations sewage sludge is not recommended to be spread on pasture.

Looking strictly to the data available, the most efficient practice to minimise the exposure to grazing cattle would be to include a treatment barrier (not including storage), apply the material through surface application and make use of a prolonged holding time. The choice of application method is not straightforward. Results from the QMRA in Paper IV showed that in term of *Salmonella* reduction, surface application of the materials is beneficial. This is mainly due to differences in environmental parameters, for example, increased contact with UV-light and increased desiccation (Hutchison *et al.*, 2004; Bolton *et al.*, 1999). However, surface application increases the exposure to wildlife and animals in the surroundings, for example by treading on fertilised land, run-off to waters and ingestion of contaminated crops.

Concerning holding time between fertilisation and grazing as a way to reduce the exposure, it must be remembered that soil reduction, in similarity with storage, is a passive process. Making use of holding times, without sanitisation treatment, between fertilisation and letting cattle onto the pasture

will eventually reduce the exposure dose to a negligible level. However, as with surface application, this approach will increase the risk for spread in the environment.

The environment plays an important role in the spread of a disease through being part of the epidemiology triangle, causing or allowing the disease to be transmitted. The choice of barriers represents a good example of the many aspects of environmental factors that need to be taken into consideration and thus the importance of understanding the environmental factors for better knowledge concerning disease transmission. All the above mentioned parameters have to be taken into account when giving recommendations and setting regulations on how to apply organic wastes and manure to land in order to reduce the risk of pathogen transmission. By choosing a prolonged holding time as the single barrier of transmission of pathogens, it is possible to get a reduced risk for exposure to grazing animals over time. However, it do not prevent transmission of pathogens to the environment, with the potential risk of further transmission to wildlife, humans and into the food/feed chain.

7.2 Considerations regarding thermal treatment as a barrier

By applying proper sanitisation treatment to the organic waste before land application the risk for transmission of pathogens to the environment can be minimised. As mentioned there are several parameters of importance for microbial inactivation or survival, *e.g.* oxygen, moisture, available nutrients, pH and competing microbiota (Jacobsen & Bech, 2012; Epstein, 1997; Haug, 1993). It must also be kept in mind that in a laboratory-scale experiment of sanitisation treatments the control of parameters is easy to handle, while in a full-scale plant this may prove more difficult. However, some parameters ought to be more easily monitored and controlled during sanitisation treatments than others, *e.g.* temperature. Hence, guidelines should be based on such relatively easily controlled parameters in order to be monitored and controlled during the sanitisation treatment. However, other parameters will affect the results of the treatment, especially when the parameter used to achieve sanitisation is close to the limit of what the organism can tolerate, such as in the case of lower temperatures during thermal treatment (Lund *et al.*, 1996). This is illustrated in Papers I and III, where an increased variation in inactivation rates are obtained at lower temperatures.

While the main goal for organic waste treatment processes such as composting and anaerobic digestion is to stabilize the material, it is important to point out that through good management of the processes, such as keeping a high and even temperature throughout the material, sanitisation of the materials

used as substrate can also be achieved. In some cases, such management may enable the exclusion of pre-treatment of organic waste and thus open the way for less costly strategies for sanitisation treatment.

Considering anaerobic digestion and composting in terms of processing of organic waste, anaerobic digestion might be preferable out of environmental and nutritional aspects such as higher plant-available nutrient content in the end-product and the lower emission of green-house gases compared with from composts. However, as a method for handling materials, *e.g.* manure, bedding material, eggs and carcasses originating from a disease outbreak, composting is a suitable treatment option. This is assuming that the compost is handled properly, paying attention to the design and monitoring of the system (Berge *et al.*, 2009).

Using part of the compost as an insulation layer, using an un-insulated or poorly insulated reactor or using air that has not been preheated for aeration of the reactor are all possible causes of psycrophilic or mesophilic zones during composting. In addition, failing to keep suitable moisture content in the compost material may result in uneven temperatures or the compost not reaching high enough temperatures. Excessive moisture in the material may block the free pores in the compost, resulting in anaerobic areas inhibiting the growth of aerobic microorganisms and thus lowering heat evolution. Low MC, on the other hand, will limit the microbial activity as a result of limiting nutrient availability and thereby also influence the heat evolution of the compost (Ceustermans *et al.*, 2010). As shown in this thesis, there is a hygiene risk associated with these colder zones. Although some pathogens, such as AIV, will be reduced even at mesophilic temperatures (Paper II), other pathogens can survive for extended periods of time (Paper I) and some bacteria might even regrow under favourable conditions (Paper III). One way of handling the problem of psycrophilic and mesophilic zones is to carry out repeated turning of the compost material in order to ensure uniformity of temperature. If the outer layer of the compost pile is assumed to keep the lowest temperature due to loss of heat to the surrounding environment, turning of the compost ensures that any material starting out in the outer layer will be treated at high temperatures. The overall best approach for achieving good hygiene standards during the composting process is for composting to be performed in insulated reactors with preheated air for ventilation to avoid the formation of psycrophilic and mesophilic zones. The use of reactors for composting also has the advantage that contact with vector animals such as birds and rodents can be minimised compared with windrow composting.

7.2.1 On-farm composting

For practical and health reasons, the recommended reduction targets recommended might need to be adjusted when handling materials from a disease outbreak, by application of even higher safety margins and additional precaution when handling the material.

Following a disease outbreak materials originating from the farm needs to undergo a sanitisation treatment before any kind of land application to limit the transmission of the pathogen to the environment. Other disposal options such as landfilling or are not appropriate for such materials due to the risk of leaching into groundwater, run-off and long survival in the environment. Furthermore, transportation of materials from disease outbreaks has been shown to be a risk factor for spread of pathogens (McQuiston *et al.*, 2005). Thus options involving transportation *e.g.* treatment at rendering plants or incineration plants might be less desirable than on-farm treatment options such as composting. In addition viable infectious pathogens may be carried in the smoke from burning of such materials and thereby cause further disease transmission (Sutmoller *et al.*, 2003). In view of this on-farm composting can be a viable option to obtain an end-product with minimal risk to human and animal health.

At present materials originating from epizootic outbreaks, ABP category 2 as defined by Regulation (EC) No. 1069/2009, may not be composted or transformed into biogas within the EU. However, several studies, including Paper II, have shown that composting of materials from epizootic outbreaks, including carcasses, can eliminate pathogens such as AIV from the materials (Berge *et al.*, 2009; Wilkinson, 2007; Bendfeldt *et al.*, 2006). Furthermore, composting of carcasses and other materials originating from AI-outbreaks has been performed successfully at several occasions, for example in Canada, where the Canadian Food Inspection Agency sees composting as one of the preferred method of disposal (CFIA, 2010; Howden, 2009; Spencer *et al.*, 2004).

When setting requirements on the treatment parameters used during sanitisation by on-farm composting following an epizootic disease outbreak, there might be a need for additional safety limits. One such requirement can be an increased reduction target before the treatment process can be approved. In Paper II a sufficient reduction was assumed to be the extrapolated value corresponding to a 12 $\log_{10}$ reduction, which presented overkill sterilization. In order to add additional safety margins, such extrapolation can be calculated using the upper limit of either the 95% or 99% confidence interval based on available data. Using the data obtained from Paper II, such reduction targets

can be reached within 7.6 h at temperatures of ≥35°C based on the upper 99% confidence interval.

It is also important to remember that regimes for handling materials originating from a disease outbreak must be able to take care of large amount of materials quickly. A high temperature throughout the whole material can be ensured by the use of a reactor with sufficient insulation or in-house composting systems. Use of such systems will lower the health risks associated with direct handling of the materials and the risk for further transmission of the pathogen associated with turning of the materials. Furthermore, the use of such systems minimises the risk for transmission of the pathogen by vector animals such as rats or birds. Alternatively materials for covering and insulation can be used. However, turning should still be avoided during the first stage of the composting process. Assuming that the surface of the compost is the part that has the lowest temperature, due to heat loss to the surroundings, monitoring of the surface temperature could be used to control the efficiency of on-farm composting.

As an enveloped virus, AIV is rather sensitive to thermal treatments compared with other viruses (Bertrand *et al.*, 2012; Wichuk & McCartney, 2007; Bachrach *et al.*, 1957). Due to the fact that when handling viruses there is no risk of regrowth of the pathogen at low treatment temperature, low temperatures can be used, ≥35°C, while still resulting in a rapid reduction in the virus. However, to achieve proper sanitisation of materials originating from disease outbreaks, caused by other viral pathogens, time and temperature combinations need to be optimised.

7.3 Guidelines to fulfil reduction targets set on ABP by EU regulations

The health status, among production animals, in Sweden today is rather good concerning pathogens such as *Salmonella* and VTEC, in comparison with that in other countries. However, it is important to take into consideration that a number of factors can affect the pathogen prevalence. Among these are environmental factors, such as climate change, leading to *e.g.* increased temperatures and drought. Pathogen-related factors, such as, genetic changes making existing species more tolerant to treatments, is another possibility. Further changes in eating habits and food processing must also be taken into consideration as possible factors affecting the risk for disease transmission (Olaimat & Holley, 2012; Fratamico *et al.*, 2008). Thus, addition of safety margins to the treatment recommendations ought to be implemented. Addition of safety margins will also cover some of the variations in inactivation rates

Table 10. *Time needed to reach reduction targets set by Commission Regulation no. 142/2011, a 5 log_{10} reduction in the bacteria Salmonella Senftenberg W775 or Enterococcus faecalis, and a 3 log_{10} reduction in the thermotolerant virus PPV. The time (h) is based on the upper 95% confidence interval limit in cattle faeces for temperatures up to 55°C and in saline solution at 70°C.*

Temperature (°C)	Bacterial reduction time (h)	Viral reduction time (h)
49	27.7	74.4
50.5	11.7	n.d.
52.0	5.85	63.9
53.5	5.3	n.d.
55.0	3.3	42.0
70.0	<0.08	4.53

that might occur as a result of differences in matrix, as previously discussed, and in thermotolerance between bacterial and viral species and subpopulations. In the present thesis the upper limit of the confidence interval (95% or 99%) was used to add a safety margin to the treatment recommendations. Keeping in mind that these recommendations give the minimum amount of time that the whole material has to maintain the given temperature, additional safety margins is added by reduction occurring during heating and cooling of the material.

The guidelines for sanitisation through thermal treatment depend on the organism studied and material, as well as the temperature and time combination of choice. The general recommendation for thermal sanitisation treatment is a temperature above 50°C. Even if some pathogens are easily inactivated at lower temperatures, such as AIV, several pathogens show slow or no reduction at lower temperatures, and some might even regrow. Table 10 list time and temperatures combinations able to fulfil the reduction targets set by the Commission Regulation (EU) No. 142/2011 on ABP category 3 materials, based on the studies presented in Paper I with regard to bacterial, *S.* Senftenberg W775 or enterococci, and viral (PPV) reduction in faecal material.

As seen in Table 10, the reduction target for thermotolerant viruses will on all occasions be that setting the treatment time. However, as stated in the regulations, viral reduction targets only need to be taken into consideration when viruses hare considered to be a risk (EU, 2011). This leaves the decision on when to take viruses into consideration is left to be decided by the relevant authority of each member country. However, as discussed in section 5.5.2, setting treatment recommendations by the reduction in PPV would result in an extremely conservative recommendation. Paper I presents the ΦX174 phage as a possible model organism for relevant but less thermotolerant viruses, which

might be a more reasonable way to validate process parameters. This approach would lower the recommended time and temperature combinations to 69.7, 13.9, 12.3, 6 and 5.8 h at 49.0, 50.5, 52.0, 53.5 and 55.0°C respectively.

As described by Bertrand *et al.* (2012) and illustrated in section 5.4, meta analysis of data from the literature might increase our knowledge about the inactivation rates of pathogens in a broader span of complex matrices. That would result in an even better projection of the treatment times needed to ensure a safe end-product and in better decision support tools in the future.

8 Conclusions

- To ensure a rapid 5 $\log_{10}$ reduction in *S.* Senftenberg W775 and *Enterococcus faecalis* temperatures above 50°C are generally recommended with a maximal treatment time of 11.7 h at 50.5°C. However, a sufficient reduction can be reached at lower temperatures (46-49°C) with significantly longer treatment time, at least 27.7 h. Reduction of porcine parvovirus (PPV) by 3 $\log_{10}$ requires much longer treatment time, 42h at 55°C, due to the high thermotolerance of the virus.

- At temperatures as high as 70°C, rapid inactivation of *S.* Senftenberg W775 and *Ent. faecalis* occurs, with >5$\log_{10}$ reduction well within 5 min. However, the time required for reaching a 3 $\log_{10}$ reduction in PPV based on the upper limit of the 95% confidence interval in saline solution is no less than 4.5 h.

- *Ent. faecalis* were shown to be a relevant process indicator for *Salmonella* spp. by exhibit a slightly higher thermotolerance during thermal treatment. However, the presence of naturally occurring thermotolerant subpopulations of *Enterococcus* spp. may pose a problem in validation of alternative treatments for ABP category 3 processing.

- Validation of thermal treatments based on viral reduction targets presents several obstacles. Due to the extremely high thermotolerance of PPV, a more suitable process indicator, such as the ΦX174 phage, should be chosen for validation purposes. In addition, during thermal treatment, part of the apparent inactivation is not true biological of the virus, but an adsorption to organic materials in the samples.

- Microorganisms with low thermotolerance, such as HPAIV, can be effectively reduced even at mesophilic temperatures (35°C) within a short period of time (<7.6h to achieve a 12 $\log_{10}$ reduction).

- At psycrophilic and mesophilic temperatures, *Salmonella* has the potential to regrow in materials from organic waste composts of low maturity.

- Sanitisation treatment of organic waste should be able to reduce the exposure dose of viable *Salmonella* by at least 10^5-fold. This is assuming that the required treatment parameters are upheld throughout the treatment.

- With a holding time of 30 days between fertilisation and start of grazing it is possible to achieve a 10^3-10^7-fold reduction in the dose of viable *Salmonella* ingested by cattle, depending on application method and soil type.

- Barriers applied after land application of organic waste can reduce the risk of grazing cattle ingesting *Salmonella*. However, in a broad perspective, such additional barriers cannot replace the importance of sanitisation treatments in reducing the risk for transmission of pathogens into the environment.

9 Future perspectives

A great deal of work has been done to date on inactivation rates for a broad span of microorganisms, in different matrices, at different temperatures and depending on a vast amount of environmental parameters affecting the survival and inactivation. Making use of the collective knowledge and investigating the possibility to develop more general inactivation models for organic waste would be of great interest and could be useful in the work of setting proper guidelines and regulations. By development of inactivation models and validated sanitisation treatments the best available data can be used as input in risk assessment in order to give better understanding of the potential effects.

In the validation process it is of great importance that the choice of process indicators is thoroughly discussed to end up with a proper model organism that is neither too conservative nor too liberal. An increased use of bacteriophages as process indicators for viruses in organic waste treatment is tempting but has to be more closely investigated. Through the use of model organisms or process indicators for viruses, expensive virus analysis can be replaced, with simple, fast and rather cheap analysis for bacteriophages. Furthermore, the inactivation of viruses needs to be further so that possible adsorption to solids in manure can be distinguished from the true inactivation, in order to ensure proper validation of sanitisation methods. In addition it must be investigated that processes able to fulfil set reduction targets will also live up to other processing standards for validation purposes, for instance concentrations of organisms allowed in the end-product.

A need for increased knowledge about the risks associated with application of materials such as manure, onto agricultural land has been expressed. There are also several other scenarios of interest to be incorporated into the risk assessment, *e.g.* spread through flooding of pasture but also other organisms of interest *e.g.* VTEC and parasites, and other animal species *e.g.* swine.

To go from estimations of the ingested dose to probability of infection proper dose-response models for cattle are needed. As adopting models from other species carries the risk of over- or underestimating the risk of adverse health effects, the possibility of developing dose-response models from available data, *e.g.* outbreak data, should be investigated.

However, there will always be gaps in data that need to be filled. At present, a lack of quantitative data on pathogens in organic waste, and their epidemiology is causing problems risk assessment approaches. Increased knowledge of the quantities of pathogens present in organic waste in Sweden would decrease the uncertainty of the analysis. A risk assessment will always be a work in progress! Following the acquisition of new knowledge, it is important that the current approaches are updated and refined so that they can provide better support to decision makers and risk managers.

10 Populärvetenskaplig sammanställning

Spridning av organiskt avfall på mark bidrar till ett kretslopp av växtnäringsämnen och tillför även organiskt material till jorden, vilket förbättrar viktiga egenskaper hos jorden. Dock kan det i organiskt avfall även förekomma sjukdomsframkallande organismer. Källan till dessa organismer kan vara sjuka djur och människor men de kan även komma från symptomfria bärare av smittämnena. Spridning av dessa fraktioner till mark kan därmed utgöra en risk för såväl människors som djurs hälsa. För att förhindra att sjukdomsframkallande mikroorganismer sprids i livsmedelskedjan kan olika typer av barriärer tillämpas. Exempel på sådana barriärer är, hygieniserande behandlingar, betesrestriktioner, skörderestriktioner samt restriktioner i val av gröda. Samtliga barriärer syftar till att minska risken för spridning av sjukdomsframkallande mikroorganismer vid kretslopp av organiskt avfall.

Genom tillämpning av hygieniserande behandlingar, t.ex. pastörisering eller ammoniakbehandlingar, kan en relativt snabb och säker reduktion av sjukdomsframkallande mikroorganismer uppnås.

Denna avhandling behandlar främst hygienisering av organiskt avfall genom värmebehandling. För en tillräcklig reduktion dvs. för att uppnå de uppställda reduktionskraven (EU-förordning No. 142/2011) för *Salmonella*, enterokocker och värmetåliga virus, rekommenderas att behandlingen sker vid en temperatur på över 50°C (Artikel I). För att undvika tillväxt i materialet av exempelvis *Salmonella* är det viktigt att undvika zoner med låg temperatur under behandlingen då tillväxt kan förekomma i dessa (Artikel III). Dock kan i vissa fall, beroende på värmetåligheten hos olika mikroorganismer, en fullgod reduktion uppnås vid betydligt lägre temperaturer än 50°C. Ett exempel på detta är kompostering av material smittat med fågelinfluensavirus för vilket en fullgod reduktion kan uppnås även vid mesofila temperaturer (35°C) (Artikel II).

I vissa fall ses även lagring som en tillämpbar hygieniserande behandlingsteknik, så som för avloppsslam i enlighet med REVAQ´s rekommendationer. Dock räknas lagring inte som en hygieniserande behandling i den här avhandlingen. Lagring är att se som en passiv process, där omgivningsfaktorer leder till att det med tiden sker en reduktion av de sjukdomsframkallande mikroorganismerna. Dock är det nästintill omöjligt att standardisera metoden i avseende att avgöra hur stor reduktion som sker inom en viss tidsrymd.

Efter spridning till mark kan många mikroorganismer överleva länge och på så vis kan smittan föras vidare in i livsmedelskedjan. I likhet med vid lagring sker en reduktion över tid till följd av en rad olika miljöfaktorer t.ex. uttorkning, pH i marken och solljus. Dock är det viktigt att vara medveten om att det är svårt att förutse hur stor och snabb reduktionen är som uppnås och variation i väderlek mellan år och säsong har därmed ett stort inflytande på reduktionshastigheten. Om ohygieniserat material sprids finns det även en risk för smittspridning i miljön genom av avrinning till vatten och översvämning av landareal. Smittan kan även innebära en risk för vilda djur och människor som vistas i området samt kan spridas vidare via vektordjur som fåglar och gnagare. Av denna anledning bör barriärer som tillämpas efter spridning av organiskt avfall t.ex. betesrestriktioner, skörderestriktioner och restriktioner i val av gröda, inte ersätta en hygieniserande behandling utan ses som ett komplement för ytterligare säkerhet (Artikel IV).

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