Pathogen Inactivation and Regrowth in Organic Waste during Biological Treatment

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Abstract

After proper sanitation treatment, organic wastes such as animal by-products, *e.g.* catering waste, slaughterhouse waste and manure can be used as fertilisers and soil conditioners and thereby contribute to a sustainable society. However, organic waste may contain pathogenic microorganisms and can thus present a health risk to both humans and animals if not properly treated.

In the present thesis regrowth potential of *Salmonella* Typhimurium, *Enterococcus* spp. and coliforms in organic waste at psychrophilic and mesophilic temperatures and the time and temperature combinations required for pathogen inactivation during thermal treatments was investigated. This was done with the aim to contribute to hygienically safe recycling of organic waste.

Pathogen growth was observed in active compost material as well as in fresh cattle manure. The growth potential decreased with increased maturity of the compost.

In thermal treatment of fresh cattle manure, a treatment temperature of 52°C and a retention time of 17.2 h or a temperature of 55°C during 16.9 h were needed to achieve the reduction targets set by current EU regulation in terms of bacterial reduction. However, this time and temperature combination was not sufficient to achieve the reduction target of 3 $\log_{10}$ for parvovirus as a thermoresistant virus.

The inactivation rate of *Salmonella* Senftenberg W775 and *Enterococcus* spp. were found to increase with increased moisture content whereas the opposite relationship between inactivation rate and moisture content was observed for viruses.

Keywords: *Salmonella* spp., *Enterococcus* spp., coliforms, bacteriophage ΦX174, PPV, sanitation, manure, household waste, thermal treatment, ABP

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"Opportunity is missed by most because it's dressed in overalls and looks like work"
Thomas Alva Edison

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 Josefine Elving, Jakob R. Ottoson, Björn Vinnerås, Ann Albihn. Growth potential of faecal bacteria in simulated psychrophilic/mesophilic zones during composting of organic waste (accepted to Journal of Applied Microbiology).

II Josefine Elving, Björn Vinnerås, Ann Albihn, Jakob R. Ottoson. Thermal inactivation of *Salmonella* and viruses in cattle manure and saline solution (manuscript)

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Notes on the authorship of papers:

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

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

Abbreviations

ABP	Animal by-products
ATCC	American Type Culture Collection
BSE	Bovine spongiform encephalopathy
CCUG	Culture Collection, University of Gothenburg
CFU	Colony-forming units
EHEC	Enterohaemorrhagic *Escherichia coli*
NCTC	National Collection of Type Cultures
NMKL	Nordic Committee on Food Analysis
P/M	psychrophilic/mesophilic
SlaBa	Slanetz-Bartley
SJV	Swedish Board of Agriculture
SMI	Swedish Institute for Infectious Disease Control
SVA	National Veterinary Institute
TSE	Transmissible spongiform encephalopathy
PFU	Plaque-forming units
PPV	Porcine parvovirus
VBNC	Viable but non-cultureable
VTEC	Verotoxin-producing *Escherichia coli*
VRG	Violet red bile agar
XLD	Xylose Lysine Desoxycholate

Definitions

Animal by-products	Defined in EC regulation no. 1774/2002 and EC regulation no. 208/2006, *e.g.* manure, food waste and slaughterhouse waste.
Arable land	Land that is used for growing crops and grazing areas
Catering waste	Defined in EC regulation no. 208/2006 as "all waste food originating in restaurants, catering facilities and kitchens, including central kitchens and household kitchens"
Coliforms	Bacteria belonging to the family *Enterobacteriaceae*
Droplet infection	Transmission of infection from one individual to another by droplets of moisture *e.g.* through sneezing or coughing
D-value	The amount of time required for a 10-fold (1 log) reduction in the number of organisms
Mesophilic temperature	15-40°C
Organic waste	Biodegradable wastes, *e.g.* animal by-products category 3, manure and sewage sludge
Pathogen	Infectious microorganism that causes illness/infection in human and/or animals
Protozoa	Single-cell organism that can only divide within the host organism
Psychrophilic temperature	<15°C
Thermophilic temperature	>45°C
Thermotolerant coliforms	Coliforms growing at 44°C
Zoonose	Infection that can spread between humans and animals

Microbial nomenclature

B. - Bacillus
Camp. – Campylobacter
Cl. – Clostridium
E. – Escherichia
Ent. - Enterococcus
L. – Listeria
Myco. – Mycobacterium
Salm. – Salmonella

Introduction

The importance of replacing nutrients and organic matter that are depleted under continuous cropping has long been known. Depending on the material used, application of organic waste to *e.g.* arable land can provide nutrients important for plant growth, including nitrogen, phosphorus, sulphur and potassium, as well as some essential micro nutrients such as nickel, zinc and copper.

Recycling of organic waste through land application serves several purposes. Reuse of organic waste helps reduce the large amounts of waste produced by society and the cost of disposal of this waste. The addition of organic matter to soils can 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. Although, application of raw waste containing organic forms of plant nutrients may not yield good results, since crops normally take up the inorganic forms of the nutrients. However, complex organic compounds can be broken down into simple organic and inorganic compounds that are available to plants through bacterial activities in *e.g.* composting or fermentation in biogas plants.

Furthermore, the use of untreated or insufficiently treated organic waste is undesirable from a public health point of view due to the risk of pathogens in the material. Zoonotic microorganisms such as *Salmonella*, *Campylobacter* and verotoxin-producing *Escherichia coli* (VTEC) have been shown to occur in organic waste. The risk of pathogens in organic waste varies depending on the incoming material, *e.g.* garden waste does not pose as high a risk as ABP. Hence the composition of the incoming material has to be taken into consideration when evaluating the risk of recycling. For example a treatment plant retrieving animal by-products must be approved by the National Board of Agriculture and use a validated treatment process.

This thesis deals with regrowth of pathogenic bacteria in compost samples of different maturities and inactivation of bacteria and virus during thermal treatment of materials with varying dry matter content.

Objectives of the present study

- To contribute to better overall understanding of hygienically safe recycling of organic waste, and thus avoid biosecurity risks for animals and humans.
- To investigate the growth potential of pathogens in organic waste depending on different physico-chemical factors.
- To determine the inactivation rate of bacteria and viruses at different temperatures and dry matter contents.

Background

Pathogens in organic waste

Pathogens present in organic waste can originate from diseased or infected but symptom-free animal or human carriers. Pathogens can be secreted with faeces and can also be present within the tissues of infected animals or on faeces-contaminated tissues. Many of the infective agents are zoonoses and are therefore of special interest when recycling organic waste between urban and rural areas.

In addition to pathogenic bacteria, virus and parasites, several 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 exposure to the recycled product.

Bacterial pathogens

In general, bacteria are more sensitive to different environmental factors and treatments than viruses and parasites. However, 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). Organic waste can contain pathogenic bacteria from several different genera, *e.g. Salmonella, Listeria, Escherichia, Campylobacter, Mycobacterium, Clostridium* and *Yersinia* (Strauch, 1991; Dudley *et al.*, 1980). Many of these are zoonoses and they can cause symptom-free infections as well as serious infections.

Salmonella spp. has been reported to survive for up to 300 days in soil treated with manure (Jones, 1986) and can grow at temperatures ranging from 6 to 47°C (Mitscherlich & Marth, 1984). In Sweden, the frequency of

salmonella-infected animals is low, which can be attributed to a strict *Salmonella* control programme in livestock.

Listeria has been shown to grow at temperatures ranging from 1 to 45°C (Junttila *et al.*, 1988), which affects the food-borne transmission of the bacteria since it is able to multiply at refrigerator temperatures. *Listeria monocytogenes* can persist for up to six months in slurry (Nicholson *et al.*, 2005) and for several weeks in compost (Lemunier *et al.*, 2005). Listeriosis is a zoonose that is usually symptom-free, although infections manifesting symptoms do occur in pregnant women and immunosupressed individuals.

Escherichia coli O157 is the serotype of *E. coli* that causes most health problems for humans. *E. coli* O157 has been shown to survive for up to 10 weeks in faeces and has also been shown to produce toxin and multiply in faeces at 22 and 37°C (Wang *et al.*, 1996). Surveys have isolated *E. coli* O157 from 8.9% of cattle herds tested in Sweden, although in southern Sweden the prevalence is as high as 23% (Eriksson *et al.*, 2005).

Data on survival of *Campylobacter* is diverse which can partly be assigned to the fact that *Campylobacter* can enter a viable but non-cultureable (VBNC) state. As a result of this they can be hard to detect in samples of organic waste with standard culture methods (Gallay *et al.*, 2006; Jones, 2001). At present, methods for isolation of the VBNC state of the bacteria are poor, making data on survival of *Campylobacter* in organic waste scarce and uncertain (Sidhu & Toze, 2009).

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). The spores are tolerant to heat and can persist in soil for decades (Mitscherlich & Marth, 1984). Endospores are not metabolically active but can under favourable conditions germinate and develop into fully functional vegetative cells.

Most *Bacillus* spp. and *Clostridium* spp. are harmless, but there are pathogenic species such as *B. anthracis, Cl. botulinum* and *Cl. chauvoei*. Other species such as *B. cereus* and *Cl. perfringens* are opportunistic pathogens and are thus usually harmless, but can cause disease under particular circumstances (Valdez *et al.*, 2009; Ozkocaman *et al.*, 2006; Gyles & Thoen, 1993).

The survival of spore-forming bacteria during processing in biogas-plants has been extensively investigated by (Bagge, 2009)

Viral pathogens

Viruses are the most common cause of foodborne gastrointestinal infections in humans world-wide (Svensson, 2000). Many viruses are transmitted through the faecal-oral route, but transmission can also occur via the respiratory route or via droplet infection. During intestinal infection viral particles may occur in high concentrations in faeces from animals and humans.

Viruses are resistant to many different types of treatments and harsh environmental conditions, *e.g.* parvoviruses and circoviruses 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 groundwater and other water reservoirs. The probability of infection is in general high at even a low dose of the virus. 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 (Anon., 1999). Viruses that are of interest as regards organic waste include Adenoviruses, Caliciviruses, Enteroviruses, Rotavirus, Hepatitis A and E viruses. All of these are non-enveloped viruses and hence more thermoresistant than enveloped viruses.

EC regulation no. 208/2006 suggests using a thermoresistant virus such as parvovirus in the validation of alternative sanitation methods. Parvovirus is a small, non-enveloped, virus that belongs to the family *Parvoviridae*, genus *Parvovirus*. Parvoviruses are known to cause infection in swine, cat, dog, mink, geese and ducks, while humans can also be infected. However, under normal circumstances parvovirus is host-specific.

Parvovirus is more stable at high temperatures than most other viruses and is therefore often used as an indicator organism in different kinds of treatments. In a laboratory-scale compost simulating operating temperatures in a full-scale compost, bovine parvovirus is reported to be inactivated within 28 days of composting (Monteith *et al.*, 1986). Haas *et al.* (1995) found that 8 days are required for inactivation of parvovirus at 55°C.

Parasites

Parasites are organisms that live in or on and take nutrients from another organism, and hence a parasite cannot live independently. Although parasite eggs, cysts and oocysts are highly resistant to harsh environmental conditions and can in some cases survive for several years in the environment. Parasites include Protozoa, Helminths and Arthropods. Some parasites are host-specific, while others are zoonoses. The lifecycle of parasites often includes

more than one host, an intermediate and a main host. The main host excretes larvae, eggs, cysts or oocysts with faeces, and these infect the intermediate host. However, some parasites have a direct life cycle and undergo their whole development in one host.

Cryptosporidium is one example of a zoonotic parasite. *Cryptosporidium parvum* is a common cause of cryptosporidiosis in humans and is also reported to infect other mammals, *e.g.* cattle (Cotruvo *et al.*, 2004; Bitton, 1999).

Prions

Prions are the cause of Transmissible Spongiform Encephalopathy (TSE) and have been found in cattle (BSE), sheep (scrapie), cats (FSE) and many other animals including humans. There are also very rare sporadic forms of prion such as Creutzfeldt-Jakob Disease in humans. This is due to a mutation in the prion gene, which makes it easier for these genes to adopt the disease form. Prions can be present in the brain and lymph tissue of infected animals, and hence these tissues are classified as specific risk material (SRM) (999/2001/EC) and have to be strictly treated as ABP category 1 materials in accordance with the ABP-regulation (Table 1).

Studies have shown that prions are not degraded during thermophilic anaerobic or aerobic treatment (Böhm *et al.*, 2008). Furthermore, prions are not denatured during pasteurisation at 70°C for 60 min nor by boiling or formalin treatment and can withstand temperatures up to 800°C as well as radiation and conventional autoclaving.

European legislation

To reduce the risk of pathogen spread through the recycling of organic waste such as ABP, sanitation treatment of the materials is required. ABP are divided into three risk categories according to the ABP-regulation (1774/2002/EC) (Table 1).

The ABP-regulation aims to avoid that material that may contain prions are introduced to recycling of organic waste. In accordance with ABP-regulation material such as animals suspected of being infected with TSE or animals killed in the context of TSE eradication measures should be processed by heating to a core temperature of more than 133°C under 3 bars of pressure by saturated steam for 20 minutes.

ABP category 3 and in some cases materials from category 2 can be used as substrate for biogas and composting plants provided that requirements set by EC no. 1774/2002 and 208/2006 are met. A common sanitation

treatment for ABP category 3 destined for composting or the biogas process is pasteurisation at 70°C for 60 min. However, alternative treatment options are permitted for ABP category 3 and manure in accordance with the ABP-regulation after approval from the relevant national authority (in Sweden the National Board of Agriculture). These alternative treatment options can differ in terms of *e.g.* time and temperature combinations, but have to meet the requirements established in EC regulation no. 208/2006.

Table 1. *Summary of the risk categorisation of animal by-products (ABP) in accordance with EC regulation no. 1774/2002 and examples of treatment requirements*

Animal by-products category	Examples of material included	Treatment requirements and use
Category 1	All body parts from animals suspected of being infected with TSE or animals killed in the context of TSE eradication, zoo animals, and pet animals.	Incineration
Category 2	Parts from ruminants except specific risk material (SRM), digestive tract contents, manure (intended for commercial purposes), animal by-products that do not belong to category 1 or 3.	Technical use Substrates for biogas production or composting after sterilisation according to EC legislation.
Category 3	ABP from animals approved for human consumption, blood (from animals other than ruminants), hides and skins, hooves and horns	Substrate for biogas-production or composting after hygiene treatment according to EC legislation

To get such approval the process has to be validated to ensure that the material at the end of the process will present a minimal biological risk. According to EC regulation no. 208/2006, alternative thermal or chemical treatment methods for ABP category 3 that is to be converted into commercial end-products through treatment in digestion or composting plants have to be able to demonstrate a 5 $\log_{10}$ reduction in *Ent. faecalis* or *Salm.* Senftenberg (775W, H_2S negative). Furthermore, a reduction of at least 3 $\log_{10}$ in the infectivity titre of thermoresistant viruses, *e.g.* parvovirus, should be demonstrated if virus has been identified as a relevant hazard. In addition to these reduction requirements, a reduction of at least 3 $\log_{10}$ viable stages in resistant parasites, *e.g.* eggs of *Ascaris* spp., is required if the process is a chemical process.

Similar requirements in terms of reduction of pathogens apply to processed manure and processed manure products for commercial use, but no reduction target is set for *Salm* Senftenberg W775.

Indicator organisms

Detection of each possible pathogen in organic waste can be troublesome. Some pathogens require expensive and time-consuming tests and can be difficult to quantify due to the need for enrichment steps during analysis and detection. As an alternative, detection and analysis of indicator organisms can be used. Indicator organisms can be used as process indicators, *i.e.* to model the behaviour of pathogens during treatment of organic waste. A suitable indicator organism should be easier to sample and measure and equally or slightly more resistant than potential pathogens. The indicator organism should preferably not be pathogenic. The most commonly used faecal indicator bacteria belong to *Enterococcus* and *Enterobacteriaceae*, although some studies have shown organisms such as coliforms and enterococci to be inadequate indicator organisms for several pathogens, especially for viruses and protozoa (Ashbolt *et al.*, 2001).

Bacteria as indicators in treatment processes

Coliforms are commonly used bacterial indicators of water and food quality. Coliforms are defined as Gram-negative, rod-shaped bacteria which ferment lactose and produce gas within 24 hours at 37°C (NMKL, 2004). Coliforms occur not only in the intestinal tract of animals but also naturally in uncontaminated soil and water environments (Berg, 1978).

One advantage in using coliforms as indicator organisms is that they occur naturally in organic waste in high numbers (10^4-10^6 CFU per gram) and thus can be analysed without additions to the material. Thermotolerant coliforms or faecal coliforms are a subgroup of coliforms defined as coliforms growing at 44°C (NMKL, 2005).

Enterococci are Gram-negative cocci that are naturally occurring in the intestinal tract of mammals. *Ent. faecalis* is one of the indicator organisms to be used for validation of sanitation processes in accordance with EC regulation 208/2006. However, little is known about the usefulness of *Ent. faecalis* as a indicator in organic waste (Sidhu & Toze, 2009). In general, enterococci are reported to be more resistant than coliforms to environmental factors such as high pH and other extreme conditions and to disinfection agents (Bitton, 1999), and thus the usefulness of enterococci in monitoring process efficiency is debatable.

Bacteriophages as indicators in treatment processes

Bacteriophages, viruses that infect bacterial cells, have a similar structure, morphology and size to many enteric viruses and they are often suggested as indicator organisms for enteric viruses (Ottoson, 2005; Moce-Llivina *et al.*, 2003; Gantzer *et al.*, 1998; Havelaar *et al.*, 1991). Like other viruses, bacteriophages are only able to multiply within a host cell.

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.* F-pili is only expressed during the bacterial growth phase of so-called F-specific RNA phages. 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 such as ΦX174, F-specific RNA (F-RNA) phages such as 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 *Salm*. Typhimurium phage 28B is not commonly found in the environment.

Somatic phages are in general more resistant to treatments than F-RNA phages (Lasobars *et al.*, 1999). Somatic phages occur in sewage sludge in concentrations of approximately 10^4 PFU per ml (Ottoson, 2005), and concentrations of 10^2-10^8 PFU per ml or gram have been reported in slaughterhouse waste and manure (Blanch *et al.*, 2004). In structural terms, F-RNA phages more similar to many infectious intestinal viruses such as Calicivirus, Enterovirus and Hepatitis virus than to the somatic coliphages, but do not occur in as high numbers.

Treatment options for organic waste

Several 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 treatment is a typical chemical treatment. However, this thesis devotes particular attention to sanitation of organic waste through the use of biological treatment methods.

Biological treatment options can be aerobic, *i.e.* composting, or anaerobic, *i.e.* anaerobic digestion. The aim of these treatments is to degrade easily available compounds and stabilise the material. In addition to this, composting also reduces the volume.

The composting process

Composting is usually performed on either solid material with a moisture content of 40-60% or on a liquid material with a moisture content of 90-98%. Chemical decomposition occurs with the help of microorganisms such as bacteria and fungi, while larger organisms such as worms and beetles are the main physical decomposers. Heat evolution during composting is caused by the microbial activity. The composting process can be divided into three phases in relation to the temperature changes within the compost - the mesophilic, thermophilic and cooling or curing phases (Epstein, 1997). During the mesophilic phase the microbial decomposition of organic matter occurs by the action of mesophilic organisms such as fungi and bacteria. As a result of active respiration, the temperature within the compost increases to levels inhibitory to mesophilic organisms but appropriate for thermophiles. During the thermophilic phase the degradation rate is high in comparison to the first phase, with optimal activity at approximately 55°C. In the third phase, the curing or cooling phase, the temperature begins to decline and a new mesophilic community develops, including fungi and Actinobacteria (actinomycetes) that can degrade more complex compounds. Macroorganisms are also active during the last phase of the composting process.

The degradation of degradable organic waste during the composting process results in a stabilisation and reduction in volume of the material into a humus-rich product called compost (Khalil *et al.*, 2001; Haug, 1993). The end-product can be used as a soil improver in commercial soil and compost products.

Temperature has been shown to be the most important parameter for inactivation of pathogens during composting (Ceustermans *et al.*, 2007; Tiquia *et al.*, 1998) and temperatures that can be reached during the composting process are lethal to many pathogenic microorganisms (Epstein, 1997). It is preferable for composting to be performed under controlled conditions, *e.g.* temperature, pH, moisture content, to ensure that a high temperature is kept in the material so that a hygienically safe end-product can be obtained.

The biogas process

The biogas process is an anaerobic process and various kinds of microorganisms participating in the process can be found in the digestor of a biogas plant. During anaerobic digestion, organic matter is degraded by bacteria in four steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1). The substrate used in a biogas plant can consist of *e.g.* household waste and ABP such as manure, slurry and blood. The incoming organic waste is minced and mixed and is then fermented anaerobically at either mesophilic (35-37°C) or thermophilic (53-55°C) temperature to produce biogas by degradation of organic waste (Hartmann & Ahring, 2006). World-wide, the mesophilic process is the more common of the two. During anaerobic digestion small amounts of heat are produced but the energy is mainly bound in the biogas produced and thus heat has to be added to the process to reach high temperatures.

The digested residues from biogas plants are rich in plant nutrients and humus and can be used as a fertiliser (Hartmann & Ahring, 2006; Gijzen, 2002). In comparison to compost the biogas digestion residues have a higher fertiliser value due to a higher content of plant-available nutrients such as nitrogen.

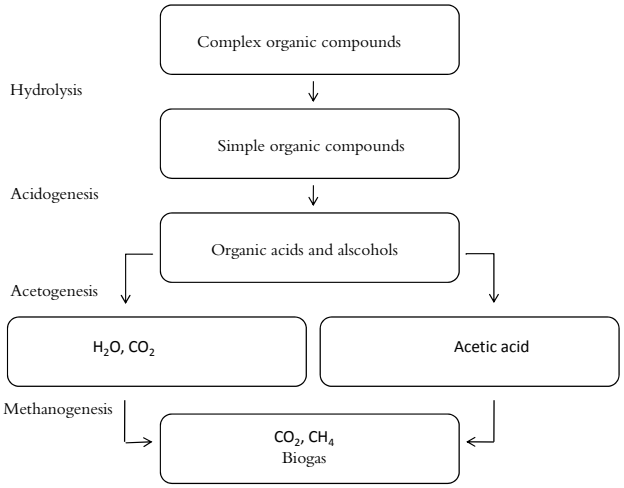

Figure 1. Schematic diagram of anaerobic digestion of organic matter showing the four steps involved in degradation.

In Sweden, most biogas plants have a separate batch-wise pasteurisation stage at 70°C for 60 min following the homogenisation phase for sanitation of the substrate. The temperature during anaerobic digestion has been shown to be the most important parameter for inactivation of pathogens (Sahlström, 2003). Inactivation of pathogens through mesophilic digestion has been shown to be poor (Yen-Phi *et al.*, 2009; Lang & Smith, 2008). Hence if relying on the process for sanitation of the substrate, thermophilic digestion where the materials are heated to high temperatures is preferable.

Table 2. *Physiochemical and biological factors that affect the survival of microorganisms. Modified from (Schönning & Stenström, 2005)*

Factor	Reaction
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 are needed.
pH	Many microorganisms are adapted to a neutral pH (7) and thus highly acidic or alkaline conditions will increase their inactivation.
Moisture	Moisture can be related to survival of the organism. In general, moisture is required for survival of microorganisms and desiccation will decrease the number of pathogens. However, in thermal treatments the conductivity increases with increased moisture, and thus also the inactivation.
Nutrients/Competing microbiota	If nutrients are available and other conditions are favourable, bacteria can multiply in the environment. 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.
Other factors	Oxygen availability, Particle size and permeability, Presence of organic and inorganic chemical compounds *e.g.* ammonia (NH_3) chemically hydrolysed or produced by bacteria can inactivate other organisms

Survival of microorganisms/pathogens in organic waste

Physiochemical and biological factors affecting microbial survival

From excretion from infected animals or humans, the concentration of microorganisms in organic waste usually declines with time. However bacteria can multiply outside the host under favourable conditions, in contrast to viruses and parasites. Some of the factors influencing the survival or die-off of pathogenic microorganisms are presented in Table 2. To devise proper treatment methods it is important to understand these factors.

Table 3. *Examples of some of D-values for pathogens and indicators in organic waste and presented in the literature.*

Organism	Temp (°C)	D-value	Source
E. coli O157:H7 in manure compost	50	135 min	(Jiang *et al.*, 2003)
Salm. Senftenberg W775 in liquid manure	50	56.7 min	(Soldierer & Strauch, 1991)
Salm. Senftenberg W775 in meat, 100% moisture	55	36 min	(Ceustermans *et al.*, 2007)
Salm. Senftenberg W775 in meat , 60% moisture	55	104 min	(Ceustermans *et al.*, 2007)
Salm. Senftenberg W775 in liquid manure	55	11.5 min	(Soldierer & Strauch, 1991)
Salm. Senftenberg W775 in sludge	55	3.2 min	(Lang & Smith, 2008)
E. coli O157:H7 in manure compost	55	35.4 min	(Jiang *et al.*, 2003)
E. coli NCTC 9001 in sludge	55	2.13 min	(Lang & Smith, 2008)
Faecal enterococci in compost	55	0.5 h	(Lund *et al.*, 1996)
Faecal coliforms in compost	55	<24 days	(Shuval *et al.*, 1991)
Bovine parvovirus in substrate to biogas plant	55	13 h	(Lund *et al.*, 1996)
Salm. Senftenberg W775 in liquid manure	60	2.3 min	(Soldierer & Strauch, 1991)
Bovine parvovirus in liquid manure	60	6 h	(Srivastava & Lund, 1980)
E. coli O157:H7 in manure compost	65	3.9 min	(Jiang *et al.*, 2003)
Porcine parvovirus in substrate from biogas plant	70	~20 min	(Sahlström *et al.*, 2008)
Bovine parvovirus in slurry	70	0.38 h	(Böhm, 2004)
Bovine parvovirus in liquid manure	70	72 min	(Srivastava & Lund, 1980)
Porcine parvovirus in substrate to biogas plant	70	60 min	(Lund *et al.*, 1996)

Pathogen persistence in organic waste

Survival of pathogens in the composting treatment has been investigated in studies of both reactor composting and windrow composting, with survival reported to occur most commonly in the latter (Wichuk & McCartney, 2007). There are several reasons for the survival of pathogens throughout the composting process and regrowth of pathogens present in amounts below the detection limit in the materials has been shown to occur under favourable conditions (Gibbs *et al.*, 1997; Burge *et al.*, 1987). Further recontamination of properly sanitised materials can occur as a result of improper handling or through vectors such as rodents and birds. Survival of pathogens in throughout the sanitation process might also be the result of insufficient time-temperature criteria for the treatment, or of the temperature not being uniform throughout the whole material due to *e.g.* poor insulation or no insulation at all. Table 3 presents examples of survival data (D-values) during thermal treatment for a number of pathogens found in organic waste.

Pathogen transmission

Transmission routes of 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 2). 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) or from garden plots fertilised with contaminated manure (Mukherjee *et al.*, 2006) and with *Salmonella* infection following irrigation of grazing areas (Jack & Hepper, 1969) or through contaminated vegetables (SMI, 2009). Further spread of pathogens can occur through vectors, *e.g.* birds, rodents and insects, that have been in contact with the contaminated site.

EC regulation no. 208/2006 aims to limit the risk of spreading ABP that has not been sufficiently sanitised and hence minimise the risk of transferring infections.

Barriers

To further minimise the risk of pathogen transmission through the recycling of organic waste, barriers or restriction can be applied to the end-products.

Barriers can be restrictions in time between fertilising land and letting animal graze the land. Such restrictions exist in the UK, where a period of two months must elapse between fertilisation of the land with compost from

catering waste, *e.g.* household waste, and grazing (Gale, 2002). However, in *e.g.* the Nordic countries, it must be borne in mind that the inactivation rate may be lower due to the colder climate. 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, fit into 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 sanitation 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 below 3 MPN g^{-1}. The Class A products can be spread without any restrictions (USEPA, 1994).

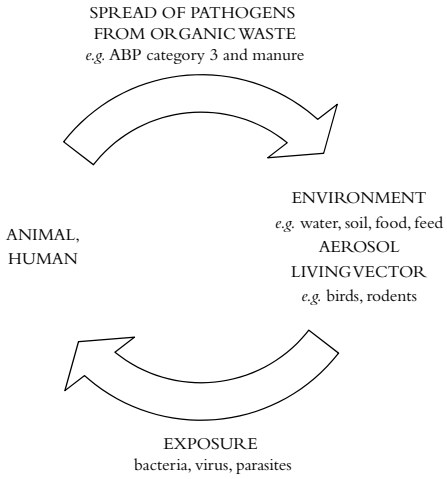

Figure 2. Infection of human and animals can occur as a result of contamination of the environment through recycling of organic waste.

Other barriers can comprise restriction of the end-use of the product through application to crops not used for food and feed if the quality of the product is uncertain. In addition to such restrictions, the choice of application method of the fertiliser can help to reduce the risk of animals coming into contact with the product.

However, when sanitation treatment of the materials is compulsory by law, as is the case with ABP category 3 and manure destined for commercial use, it must be remembered that the use of barriers is not a replacement for sanitation treatment, but merely a complement.

Comments on Materials and Methods

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

Experimental set-up

Growth and inactivation in psychrophilic/mesophilic zones (Paper I)

In Paper I compost material was 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 manure 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 and volatile solids. Samples were inoculated with *Salm.* Typhimurium, phage type 178 (isolated from sewage sludge by Sahlström *et al.* 2004), *Ent. faecalis* (ATCC 29212) and *E. coli* (ATCC 35218) and then 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.

In Paper I, a student's t-test was performed to compare the bacterial count on day 1 with that after 8 days of incubation. Pearson Product Moment Correlation was also used to analyse possible correlations between $\log_{10}$ changes in numbers of *Salmonella* and indicator organisms, moisture content, volatile solids and pH. Correlations between bacterial count and temperature, Solvita® index and Rottegrad index were analysed using Spearman Rank correlation. SigmaStat 3.0 (SPSS inc., Chicago, IL) was used for all statistical analyses in Paper I.

Thermal inactivation (Paper II)

In Paper II, inactivation of pathogens in manure was investigated using cattle manure collected from a dairy herd held indoors at Kungsängens research station, SLU, Uppsala. The manure was used as fresh cattle manure (FCM) but also as dried cattle manure (DCM). For DCM, the water content was set by drying at 37°C to a dry matter content of 25%. Both materials were analysed for *e.g.* pH, dry matter content, volatile solids and C/N ratio. Furthermore, saline solution (NaCl) was used as a third material to investigate the inactivation in a material with approximately 100% moisture content. Samples of NaCl, FCM and DCM were inoculated with *Salm.* Senftenberg W775 (NCTC 9959), *Ent. faecalis*, bacteriophage ΦX174 and porcine parvovirus (PPV). Inoculated samples were treated at 49, 52 or 55°C during 8 hours using digital block heaters (Grant Instruments, LtD, Shepreth, Cambridgeshire, England). For samples with NaCl, thermal treatment at 70°C was included.

In Paper II, general linear modelling (GLM) was performed in order to visualise interaction effects between the parameters studied. Assuming first order kinetics, linear regression analyses were performed using Minitab 15 (Minitab Ltd., Coventry, U.K.). From the regression functions obtained from the data sets, D-values with a 95% confidence interval ($CI_{95\%}$) were calculated.

Measurement of maturity

Compost stability is recognised to be 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 I, 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]

[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°C

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 lid was closed tightly. During the test the gel on the paddle did not come into contact with the sample. After 4 hours 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 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 9 being "finished" compost.

Culture and analysis of microorganisms

Bacterial organisms

For Papers I and II, bacterial microorganisms were cultured in nutrient broth in overnight cultures at 37°C. To avoid interference from other bacteria, the different bacterial strains were cultured in separate flasks before inoculation of the materials. Before plating, the samples were serially diluted in saline solution to appropriate concentrations.

Salmonella was isolated on Xylose-Lysin-Deoxycholate (XLD) agar containing 0.15% sodium-novobiocin. In brief, 0.1 ml of diluted sample was surface-spread on XLD agar and the agar plates were incubated at 37°C for 24±2 h (Papers I and II). In Paper I, colonies exhibiting a typical growth with black centre were recorded as *Salmonella*. The *Salm*. Senftenberg W775 used in Paper II is a H_2S-negative strain and thus no black centre occurs when culturing on XLD. For confirmation of *Salm*. Senftenberg W775, a MUCAP-test for confirmation of *Salmonella* spp. was used.

For analysis of *Enterococcus* spp. Slanetz-Bartley (SlaBa) agar was used. In brief, 0.1 ml of the diluted sample was surface-spread and the agar plates were incubated at 44°C for 48±4 h. Colonies exhibiting a typical growth was registered as enterococci. When recording *Enterococcus* spp., no differentiation was made between added *Ent. faecalis* and indigenous *Enterococcus* spp. (Papers I and II).

Analysis of total coliforms in Paper I was performed as follows: 1 ml of the diluted sample was mixed in a layer with violet-red bile (VRB) agar. After solidification, a second layer of VRB was added and the plates incubated at 37°C for 24±2 h. After incubation colonies exhibiting typical growth were counted and confirmation tests performed.

Bacteriophages

Bacteriophage ΦX174 was propagated in nutrient broth against its host strain *E. coli* (ATCC 13706). The bacterial host strain was killed using chloroform, the phage solution was centrifuged at 2000 x g and supernatant was collected.

Enumeration of the bacteriophages was performed according to the double agar layer method (Adams, 1959) with the host strain.

Viruses

The PPV strain 893/6 (originally isolated at the Danish Institute for Food and Veterinary Research, Lindholm, Denmark) was grown in PK-15 (pig kidney) cells as described by Sahlström *et al.* (2008).

Before enumeration of viruses in treated samples, the samples containing manure were centrifuged and filtered to remove organic material and debris. Virus titre was determined by $TCID_{50}$ quantal assay (Kärber, 1931) and to visualise the infected cells prior to microscopy an immunoperoxidase assay (IPX-assay) was performed as described in Paper II.

Main results

Growth of pathogens in organic waste at psychrophilic and mesophilic temperatures (Paper I)

The investigation of regrowth potential in compost revealed that growth of pathogens can occur in household waste compost of low maturity (Paper I). Furthermore, growth was found to occur in fresh cattle manure but not in samples collected from cattle manure compost. Samples collected from manure compost had a higher degree of maturity in comparison with the varying maturity of the samples collected from municipal composts for household waste (Table 4). Growth and inactivation of *Salm.* Typhimurium in samples from household waste compost, manure compost and fresh manure are summarised in Table 4. Similar growth and inactivation patterns were observed for *Enterococcus* spp. and coliforms. Significant negative correlations were found when comparing the growth/inactivation of the three bacteria over 8 days to the temperature of the simulated P/M zones. However, significant negative correlations between the bacterial count and maturity of the compost (Solvita® maturity index) were only found for *Salm.* Typhimurium and coliforms.

Thermal inactivation in manure (Paper II)

Pasteurisation at 70°C

Pasteurisation at 70°C proved to be effective in reduction of the bacteria *Salm.* Senftenberg W775 (NCTC 9959) and *Ent. faecalis* (ATCC 29212) and the bacteriophage ΦX174. Neither of the bacteria could be detected after 15 min of thermal treatment in saline solution. Inactivation of bacteriophage ΦX174 occurred at an even higher rate and was complete within 10 min of thermal treatment (Table 5). Thus pasteurisation at 70°C for 60 min was

found to be sufficient to reduce *Salm*. Senftenberg W775 and *Enterococcus faecalis* by 5 $\log_{10}$ cfu within 60 min. Furthermore, a 3 $\log_{10}$ reduction in bacteriophage ΦX174 was reached well within 60 min. However, inactivation of PPV was found to be low (Table 5) and a D-value of 1.2 h ($CI_{95\%}$ 1.19-1.29 h) was calculated from the data obtained, resulting in a required retention time of approximately 4 h at 70°C to obtain a 3 $\log_{10}$ reduction in PPV.

Table 4. *Maturity (Rottegrad and Solvita®) in samples of household waste compost (A1-3 and B1-3), beef cattle manure compost (C1-6) and fresh dairy cattle manure (D1). Results of comparison of the bacterial count after 8 days of incubation with bacterial count of Salm. Typhimurium 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 are indicated in bold*

Sample no.	Maturity		*Salm*. Typhimurium		
	Rottegrad	Solvita®	14°C	24°C	37°C
A1	III	4	**1.1****	**1.8*****	-1.9**
A2	IV	4	**0.5**	**1.4****	**0.9****
A3	III	5	-1.0*	-1.4**	-1.9***
B1	IV	6	**0.7**	**1.1***	**0.4**
B2	IV	8	-3.2**	-3.4	-4.3***
B3	II	3	-0.7*	-0.8**	-1.5
C1	IV	5	-1.4***	-2.1***	-2.4***
C2	V	7	-0.5***	-0.8***	-3.0**
C3	V	7	-0.4**	-0.9**	-2.2**
C4	V	8	-0.5*	-0.8**	-2.8***
C5	V	8	-1.1*	-1.4**	-2.6***
C6	V	8	-1.2***	-1.7***	-2.4***
D1	-	4	**0.3**	**0.5***	-0.8

Table 5. *Content (log_{10} CFU g^{-1} ± SD) of Salmonella Senftenberg W775, Enterococcus faecalis, bacteriophage ΦX174 and PPV in saline solution before thermal treatment and after 5, 10, 15 and 60 min at 70°C*

Organism	Before heat treatment	70°C 5 min (n=3)	70°C 10 min (n=3)	70°C 15 min (n=3)	70°C 60 min (n=3)
Salm	7.5±0.1	2.0±0.5	1.1±0.2	n.d.	n.d.
Ent	6.4±0.1	1.4±0.6	1.3±0.5	n.d.	n.d.
Phi	5.7±0.1	-	n.d.	n.d.	n.d.
PPV	6.7±0.4	-	-	6.4±0.1	5.8±0.1

n.d., not detected

Table 6. Linear regression models for Salm. Senftenberg W775, Enterococci spp., bacteriophage ΦX174 and PPV in saline solution (NaCl), fresh manure (FCM) and dried manure (DCM) at 49, 52 and 55°C and the time needed for a 5 or 3 log_{10} reduction based on the upper limit of the 95% CI.

	Temp (°C)	k	SE t	n	p	D ($CI_{95\%}$) (h)	5 log_{10} (h)	k	SE t	n	p	D ($CI_{95\%}$) (h)	3 log_{10} (h)
		Salm. Senftenberg W775						Bacteriophage ΦX174					
NaCl	49	0.235	0.021	26	<0.001	4.3 (3.6-5.2)	26.1	0.038	0,010	18	0,002	26.5 (16.8-61.6)	184.4
	52	0.683	0.039	27	<0.001	1.5 (1.3-1.7)	8.3	0.092	0,010	21	<0.001	10.9 (8.8-14.1)	42.3
	55	1.860	0.346	16	<0.001	0.5 (0.4-0.9)	4.5	0.469	0,014	18	<0.001	2.1 (2.0-2.3)	6.8
FCM	49	0.066	0.014	26	<0.001	15.1 (10.6-26.2)	131.1	0.042	0,007	21	<0.001	23.9 (17.9-36.0)	107.9
	52	0.611	0.035	25	<0.001	1.6 (1.5-1.9)	9.3	0.037	0,008	20	<0.001	27.0 (18.3-51.4)	154.1
	55	0.961	0.064	20	<0.001	1.0 (1.0-1.1)	5.6	0.108	0,007	21	<0.001	9.3 (8.2-10.7)	32.0
DCM	49	0.131	0.012	26	<0.001	7.6 (6.4-9.5)	47.4	0.222	0,028	21	<0.001	4.5 (3.6-6.1)	18.4
	52	0.224	0.021	27	<0.001	4.5 (3.7-5.5)	27.7	0.291	0,034	21	<0.001	3.4 (2.8-4.5)	13.6
	55	0.161	0.028	27	<0.001	6.2 (4.6-9.7)	48.4	0.263	0,035	21	<0.001	3.8 (3.0-5.3)	15.8
		Enterococcus spp.						PPV					
NaCl	49	0.123	0.020	27	<0.001	8.1 (6.1-12.2)	61.1	0.016	0,015	20	0,311	-	-
	52	0.534	0.043	25	<0.001	1.9 (1.6-2.2)	11.2	0.050	0,018	18	0,014	20.2 (11.4-86.7)	260.1
	55	0.886	0.128	20	<0.001	0.7 (0.5-0.9)	4.7	0.052	0,022	18	0,029	19.2 (10.2-163.2)	489.5
FCM	49	0.064	0.023	26	0.012	15.7 (8.9-65.0)	325.2	0.078	0,021	21	0,001	12.8 (8.2-28.7)	86.0
	52	0.372	0.042	26	<0.001	2.7 (2.2-3.4)	17.2	0.099	0,021	18	<0.001	10.1 (7.0-18.6)	55.7
	55	0.509	0.102	20	<0.001	2.0 (1.4-3.4)	16.9	0.084	0,024	18	0,003	11.9 (7.5-29.1)	87.3
DCM	49	0.088	0.028	27	0.005	11.4 (6.8-33.7)	168.7	0.097	0,022	21	<0.001	10.3 (7.0-19.5)	58.6
	52	0.177	0.030	27	<0.001	5.6 (4.2-8.7)	43.7	0.154	0,035	21	<0.001	6.5 (4.4-12.4)	37.3
	55	0.091	0.027	27	<0.001	11.0 (6.7-31.6)	157.9	0.207	0,036	21	<0.001	4.8 (3.5-7.6)	22.7

Thermal inactivation at 49, 52 and 55°C

Data from the investigation of thermal inactivation at 49, 52 and 55°C in manure, FCM and DCM, and NaCl are summarised in Table 6.

In general, inactivation of bacteria in NaCl and FCM at 49°C occurred at a significantly lower rate than that at 52 and 55°C, while inactivation of bacteriophage ΦX174 showed a significantly higher inactivation rate at 55°C compared with the two lower temperatures. The uncertain inactivation at 49°C for bacteria and 49 and 52°C for bacteriophage ΦX174 is reflected in the larger confidence intervals. In contrast to NaCl and FCM, no difference between treatment temperatures could be found for any of the two bacteria or for bacteriophage ΦX174 in DCM.

D-values for inactivation of PPV were not significantly different between temperatures in the different materials.

Comparisons of the dry matter content of the samples revealed increased bacterial inactivation with decreasing dry matter content, while viral inactivation increased with increasing dry matter content

Indicator organisms (Papers I and II)

During incubation at psychrophilic and mesophilic temperatures, significant correlations were found between growth and inactivation of *Salm.* Typhimurium, *Enterococcus* spp. and total coliforms (Paper I).

The use of *Enterococcus* spp. and bacteriophage ΦX174 (13706-B1), a somatic coliphage, as indicators for inactivation of *Salm.* Senftenberg and PPV during thermophilic inactivation in organic waste were evaluated in Paper II. The confidence intervals listed in Table 6 show that D-values for bacteriophage ΦX174 and PPV do not differ significantly in most datasets. However, in general the confidence intervals obtained for PPV are rather large in comparison with that of bacteriophage ΦX174. As for *Salm.* Senftenberg W775 and *Enterococcus* spp., correlations were found at 49°C in FCM as well as in DCM. At 52 and 55°C, correlations were observed between the two bacteria studied in NaCl and DCM, but not in FCM where the D-values obtained for *Enterococcus* spp. were higher than for *Salm.* Senftenberg W775.

Discussion

Growth in organic waste at psychrophilic/ mesophilic temperatures

Many studies have shown inactivation of microorganisms to be negligible or slow at low temperatures *e.g.* during mesophilic digestion (Yen-Phi *et al.*, 2009; Lang & Smith, 2008; Lund *et al.*, 1996; Kearney *et al.*, 1993). Additionally, growth of pathogens has been shown to occur in organic waste of different origins under favourable conditions such as available nutrients and not prohibitively high temperatures (Lang & Smith, 2008; Ceustermans *et al.*, 2007; Sidhu *et al.*, 2001; Sidhu *et al.*, 1999; Gibbs *et al.*, 1997; Skanavis & Yanko, 1994). Problems with regrowth and non-uniform temperatures during composting have also been cited by Wichuk and McCartney (2007) as a possible explanation for differences between expected inactivation and the actual inactivation in sanitation processes.

In Paper I we investigated the growth potential of *Salm*. Typhimurium, total coliforms and *Enterococci* spp. in organic waste compost when incubated at psychrophilic and mesophilic temperatures. In the present study growth occured at both psychrophilic (14°C) and mesophilic temperatures (24 and 37°C) in active household waste compost and in fresh cattle manure. However, as can be seen in Paper II, no growth of pathogens could be detected from 49°C upwards.

In our growth and inactivation studies of *Salm*. Typhimurium, a correlation was found to Enterococcus spp. and coliforms (Paper I). This indicates that *Enterococcus* spp. and coliforms can be used as indicators for growth of pathogens such as *Salmonella* spp. in organic waste. These findings are in line with Pourcher *et al.* (2005), who reported similar evolution of *Enterococci* and *E. coli* during composting of sludge in straw mixture. Others

have shown *Enterococci* to be more thermoresistant than coliforms (Tiquia *et al.*, 1998).

The regrowth potential of *Salm.* Typhimurium and coliforms in Paper I was significantly correlated to the maturity of the compost samples with a decreased regrowth potential with increasing maturity. This can be attributed to a lower content of available nutrients present in more mature compost, which has previously been shown to influence the regrowth potential (Inbar *et al.*, 1990). However, no distinct limit for growth could be set in relationship to the maturity of the compost samples. In general, growth was seen to occur in household waste compost with a Solvita index of 4 (defined as active compost in the Solvita® manual), while not in samples with a Solvita index of 7 or 8 (defined as finished compost in the Solvita® manual).

Regrowth was observed over 8 days at all three temperatures (14, 24 and 37°C) although the regrowth decreased with increased temperature. The fact that a regrowth potential may exist in compost material suggest that if parts of composts are within the P/M zones and pathogen growth occurs during the composting, an increase in the amount of pathogens may occur in terms of the pathogen content in the whole compost. For example, if 10% of the compost material is within a zone that can be classified as psychrophilic or mesophilic and if a growth of approximately 2 $\log_{10}$ of *Salmonella* spp. occurs within these parts of the material, as was shown to be the case during incubation of household waste compost over 8 days at 24°C (Solvita® maturity index 4) (Paper I), this will result in a 1 $\log_{10}$ increase in total amount of pathogens, even if all pathogens are inactivated in the remaining 90% of the compost. The assumption that 10% of the material is within colder zones is not an overestimation, since the amount of material in these zones can be as high as 35 %, depending on the size and profile of the compost (Haug, 1993). Additionally, too large P/M zones require increased number of turnings of the compost to reach the appropriate sanitation level.

This risk has to be taken into account when calculating the time needed for sanitation of compost material. In a worst case scenario inactivation of pathogens can start from higher levels than expected, ultimately resulting in a lower inactivation of pathogens than expected. Thus it is important to monitor the temperatures during the composting process in order to ensure that the inactivation of pathogens really fulfils the inactivation requirements established to reach the appropriate hygienic standards and minimise the risk of transmission of pathogens. This has to be taken into account in a validation, to set appropriate time and temperature combinations in terms of

pathogen reduction. In the case of virus and parasite, regrowth is not a problem. Thus, the risks with P/M zones in compost of organic waste are mainly associated with bacterial pathogens.

The advantage of reactor composting

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 can all be the cause of P/M zones during composting. As shown above, there is a hygiene risk associated with these colder zones and the overall best solution is for composting to be performed in insulated reactors with preheated air for ventilation to avoid the formation of P/M zones. The use of reactors for composting also has the advantage that contact with vectors such as birds and rodents can be minimised.

Further it is important to separate the treated and sanitised material from the incoming material, which potentially contains pathogens, to avoid recontamination of the end-product. However, this is not part of the sanitation process but of the approval process for both composting and biogas plants.

Time and temperatures needed for inactivation of pathogens

If the goal is thermal inactivation of pathogens, it is particularly critical to obtain a high temperature within the whole material and to avoid colder zones during composting of organic waste, as we showed in Paper I. In this aspect, heat generation and transport of heat are critical factors. The distribution of heat in the materials is a complex process and can be influenced by the moisture content of the material among other parameters. An increase in the moisture content results in increased conductivity of the material, so increased moisture content ought to facilitate the heat transfer within the compost.

Large amount of data on thermal inactivation of microorganisms is available in the literature (Ceustermans *et al.*, 2007; Droffner & Brinton, 1995; Burge *et al.*, 1981). Although the variation between time and temperature combinations for sanitation is large and it is not always easy to compare data from different sources. One major problem when comparing data is that many of the published studies do not give the inactivation rate for the pathogen used. In some cases it is possible to calculate the inactivation rate from the data given, but this is not always the case. Even if the time to inactivate a pathogen is a function of the inactivation rate, this does not tell us much, since the time for inactivation will increase with

increasing numbers of organisms at the start. Thus, comparison of different studies is simplified through the use of consistent parameters such as decimal reduction times (D-values).

The fact that inactivation of pathogens under controlled laboratory conditions has in some cases been shown to require quite a long time suggests that this might also be the case in full-scale plants. For example, Droffner and Brinton (1995) suggest that at least 3 days at 55°C is needed for sufficient pathogen inactivation and Burge *et al.* (1981) state that a minimum temperature of 55°C for 2.5 days is required to reach a level acceptable for commercial products from compost. These times are quite long compared with thermal treatment of fresh manure at 55°C in 16.9h, which we found to be sufficient to achieve a 5 $\log_{10}$ reduction in *Salm.* Senftenberg and *Enterococcus* spp. (Paper II).

Part of the reason for differences between time requirements presented in the literature can be explained by the dry matter content of the materials. As mentioned above the conductivity of materials increase with increased moisture content and hence also the inactivation rates ought to increase. Commercial compost usually have a dry matter content of 35-50%, which is higher than the 12% dry matter content in the manure used in Paper II. In general increased moisture content can be linked to a decreased D-value, which can be seen if comparing the D-values in several studies with materials of different dry matter content *e.g.* D-values at 55°C for *Salm.* Senftenberg W775 of 36 min at a moisture content of approximately 100% (Ceustermans *et al.*, 2007), 60 min ($CI_{95\%}$ 1.0-1.1 h) at a moisture content of 12% (Paper II), and 104 min at a moisture content of 60% (Ceustermans *et al.*, 2007).

An additional reason for the variation might lie in the fact that the material may not be homogeneous *e.g.* compost is known to be a heterogeneous material. Furthermore, the lack of data on the fraction of the composts that is within the P/M zones during the process can result in differences between studies. In addition, factors such as differences in pH and other physico-chemical parameters as well as differences and uncertainties in sampling and laboratory procedures can contribute to the differences observed.

Thermophilic treatments

For pathogen inactivation at lower temperature, an increased time is needed to reach the statutory requirements. Our results indicate that a time of 17.2 h at 52°C or 16.9 h at 55°C can be sufficient to reach the reduction targets set by EC legislation based on the inactivation of *Enterococcus* spp. in FCM.

This interval would also be sufficient for a 5 $\log_{10}$ reduction in *Salm.* Senftenberg W775

The inactivation rate for *Salm.* Senftenberg W775 and *Enterococcus* spp. in FCM and NaCl was significantly lower at 49°C as compared to 52 and 55°C. However, no significant difference could be observed between D-values in 52 and 55°C. Thus, a raise in process temperature from 52°C to 55°C seem to have only marginal impact on the time required to reach the required reduction of 5 $\log_{10}$ (Paper II). However, lowering the temperature to 49°C lead to a required time of more than 100 h. Hence, the increase of 3°C from 49°C to 52°C can be seen as a good investment in terms of the time required for sanitation during thermal treatment. These results supports the general assumption that sanitation through thermal treatments is best performed at temperatures above 50°C (Haug, 1993).

The effect of increased moisture content is evident at 52 and 55°C for both *Salm.* Senftenberg W775 and *Enterococcus* spp. with increasing inactivation at higher moisture content. However, this does not come as a surprise since the increased moisture content was expected to increase the conductivity of the material and hence the effect of temperature on the organisms as mentioned above.

In contrast to bacterial inactivation, the above mentioned time and temperature combinations of 17.2 h at 52°C or 16.9 h at 55°C are not sufficient to achieve a 3 $\log_{10}$ reduction in either bacteriophage ΦX174 or PPV. Thus, if virus has been identified as a relevant risk the time and temperature interval to fulfil the requirements set by the EC regulation would have to be based on 3 $\log_{10}$ reduction of the virus. In the case of the thermoresistant virus PPV in fresh cattle manure, this would mean prolonging the time to approximately 56 h at 52°C (Paper II).

In contrast to bacterial inactivation the inactivation rate of bacteriophage ΦX174 and PPV, decreased with increased moisture content. This was not what we had expected and the cause of this remains unclear.

Turner and Burton (1997) have suggested thermal treatment to be one of the treatment options best suited for inactivation of viruses, since solids within the material do not protect the viruses against heat. They also suggest that solids within the material can lead to increased heat retention in the material, thus leading to increased inactivation of microorganisms.

Others have suggested adsorption of virus to solids to interfere with virus inactivation (Gassilloud & Gantzer, 2005; Gerba, 1984). Thus it is possible that the increased inactivation with increased dry matter content is not a result of actual inactivation but a result of increased adsorption to solids in the manure samples. Adsorption of viruses to soil particles has been shown

to depend upon the virus being studied, *e.g.* the isoelectric point of the virus, and the materials being studied, *e.g.* organic matter in samples is believed to compete with virus for adsorption to solids (Gerba, 1984). Thus the use of supernatants from centrifuged samples to analyse the inactivation of viruses might be misleading if the virus is adsorbed and hence removed with the pellet (Haas *et al.*, 1995).

Several inactivation studies have shown a tailing or biphasic inactivation during thermal inactivation of virus with an initial high inactivation rate followed by a lower inactivation rate (Lund *et al.*, 1996; Bøtner, 1990). A possibility is that the initial observed high inactivation rate is a combined effect of inactivation and adsorption of virus to the solids. In our studies in Paper II no pronounced tailing was seen in the inactivation of PPV or bacteriophages ΦX174. However, a tendency to biphasic inactivation could be observed for both viruses, but most pronounced in DCM. If the increased inactivation in the initial stage is a result of adsorption to solids the D-value for inactivation should be calculated from the latter phase instead of calculation of the D-value including all data points, as is the case for D-values presented in Paper II. Thus, adsorption might explain the increased inactivation rates in DCM and FCM as compared to in NaCl. This has to be taken into consideration when setting appropriate time and temperature intervals to reach required sanitisation levels for virus.

However, in Paper II, the inactivation of virus was only studied during 8h and it is possible that the occurrence of these phenomena could not be clearly seen as a result of too short incubation times. Thus, it would be interesting to increase the incubation times to see if a biphasic inactivation occurs in FCM and DCM.

Different methods can be used to avoid the adsorption of viruses to solid particles, *e.g.* plastic carriers to which the virus is adsorbed, and different methods for reisolation of the virus from the supernatant. For example Bøtner (1990) showed that adding 10% fetal calf serum to the adsorption medium allowed up to 100% of the virus to be eluted. The latter method was used in Paper II, the samples were diluted in NaCl containing 10% FBS before freezing. However, the results from our studies indicate that adsorption of virus to solids in the manure might still be a possibility.

Phages as indicators for viruses during thermal inactivation

The analysis of viruses in environmental samples can be difficult and rather expensive. Bacteriophages, on the other hand, are easy and cheap to analyse and thus it is desirable to use them as process indicators for viruses.

As regards finding a bacteriophage to use as indicator organisms for PPV in sanitation treatments, Sahlström *et al.* (2008) concluded that the *Salmonella* phage 28B has an even higher thermoresistance than PPV and therefore is not suitable as an indicator organism for PPV. Hence the use of less thermoresistant bacteriophages as indicators for PPV has been suggested.

In Paper II the inactivation of bacteriophage ΦX174 was analysed and compared to the inactivation of PPV. The difference in inactivation rate was most pronounced in NaCl at 55°C, were a significant difference could be found between the D-value of 2.1h measured for bacteriophages ΦX174 and the D-value obtained for PPV was 19.2h. In none of the remaining datasets significant differences could be seen between the inactivation rates of the two viruses. Although the D-values obtained for bacteriophage ΦX174 and PPV did not differ significantly, this might be a result of large confidence intervals or other factors interrupting the analysis e.g. adsorption. Further it is interesting to note that the inactivation rate of bacteriophage ΦX174 increased significantly when the temperature was raised from 52°C to 55°C, in NaCl and FCM, while this was not the case for PPV at the temperatures used in Paper II. Hence, these results further emphasise just how thermoresistant PPV is.

Coliphages and other phages of the Enterobacteriaceae group can be expected to be present in sufficient amounts in organic waste. However, they may under extreme circumstances be able to multiply in the environment (Muniesa & Jofre, 2004). F-RNA phages do not commonly multiply in the environment, but have been reported to have a lower thermoresistance than somatic coliphages (Lasobars *et al.*, 1999). Others have reported the thermoresistance of somatic and F-RNA bacteriophages to be similar at 60°C. Furthermore, both somatic and F-RNA bacteriophages have been found to be more thermoresistant than rotavirus, poliovirus and coxsackievirus (Moce-Llivina *et al.*, 2003). This suggests that somatic coliphages and F-RNA bacteriophages could be relevant indicator organisms for viruses in a validation process. However, further research is needed to evaluate the use of bacteriophages as process indicators for viruses.

Pasteurisation at 70°C

At temperatures as high as 70°C, the survival time of most pathogens is short (Sahlström *et al.*, 2008; Jones & Martin, 2003). As we have shown (Paper II), inactivation of *Salm.* Senftenberg W775 and Enterococcus faecalis is obtained within 15 minutes at 70°C. In the same study, inactivation of the bacteriophage ΦX174 was obtained within 10 minutes. However, inactivation of PPV was poor and a D-value of 1.2 h ($CI_{95\%}$ 1.19-

1.29) was obtained in our study. PPV is known to be an extremely thermoresistant virus (Lund *et al.*, 1996; Bøtner, 1990). This is in contrast to most other viruses, which are inactivated in about 25 min at 70°C (Day & Shaw, 2001).

In a study of virus inactivation in slurry, (Bøtner, 1990) compared the inactivation of PPV to that of several other viruses, *e.g.* Aujeszky´s disease virus, classical swine fever virus and swine influenza virus, at temperatures ranging from 5-55°C. The results not only showed a temperature dependence of the inactivation rates but also large differences in thermoresistance of these viruses. While inactivation of most of the other viruses tested required a treatment time of <1 h at 55°C (initial concentration varying from $10^{4.8}$–$10^{5.8}$, detection limit of 0.7 $\log_{10}$ $TCID_{50}$/50µl), a time of 8 days was required for a 5.3 $\log_{10}$ reduction in PPV. Hence the results from pasteurisation of PPV in Paper II are not surprising.

The use of PPV is well motivated in comparative studies of different thermophilic inactivation treatments due to the fact that quite long inactivation times can be studied. Furthermore, it can be stated that if a sanitation treatment can fulfil the requirements of a 3 $\log_{10}$ reduction in parvovirus, most other viruses will be inactivated. However, to require a 3 $\log_{10}$ reduction in parvovirus is to set very high demands on the sanitation process and in comparison with *e.g.* bacteria and other viruses, it might be seen as an unnecessarily high safety margin.

Lund *et al.* (1996) suggest that when setting demands on sanitation, 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. However, in the current EC legislation parvovirus is mentioned as an example of a thermoresistant virus and hence other viruses could be used instead in the validation of sanitation treatments. Furthermore, validation of virus inactivation is only to be done when these are deemed to be a hazard. Hence it can be discussed when the time and temperature combinations set for sanitation treatment processes has to be based on a 3 $\log_{10}$ reduction of thermoresistant viruses. Thus, a risk assessment of the incoming materials, to be used as substrates in a biogas or composting plant, must be done to evaluate if viruses is a relevant hazard. If virus is not identified as a relevant hazard the time and temperature combinations should be based on a 5 $\log_{10}$ reduction of bacterial pathogens, which can be achieved in rather short time in comparison to a 3 $\log_{10}$ inactivation of such a thermoresistant virus as PPV. Further, there is no risk of regrowth of virus.

The significance of good management practice

There are several parameters of importance for the composting process, such as oxygen, moisture, available carbon and nitrogen, pH and competing microbiota (Epstein, 1997; Haug, 1993; Inbar *et al.*, 1990; Hussong *et al.*, 1985). For example, excessive moisture in the material may block the free pores in the compost, resulting in anaerobic area inhibiting the growth of aerobic microorganisms and thus lower heat evolution. Low moisture content, on the other hand, can limit the microbial activity as a result of limiting nutrient availability and thereby also influence the heat evolution of the compost. Both these scenarios would lead to a prolonged treatment period.

In a laboratory-scale experiment the control of parameters is easy to handle while in a full-scale plant this may be more difficult. However, some parameters such as temperature ought to be more easily controlled than others. Hence, one approach in setting requirements to reach sanitation in organic waste treatments would be to set simple guidelines for inactivation based on relatively easily controlled parameters such as temperature.

Even if the main goal for organic waste treatment is to stabilise the material, it is important to remember that through good management of the processes, sanitation of material can also be achieved as a result of thermal inactivation of pathogens and in these cases the pre-treatment of ABP category 3 and manure can be excluded. If the composting process is performed in well insulated reactors, the heat produced in the process can be used for sanitation of the organic waste and the same goes for pasteurisation if temperatures reached during treatment are high enough to sanitise the material.

To achieve a hygienically safe end-product, recommended time and temperature combinations for inactivation of pathogens in organic waste can be set up for composting and for biogas plants. In Paper II is was shown that a time and temperature combination of 16.9 h at 52°C or 17.2 h at 55°C is enough to reach requirements set for inactivation of bacteria in FCM. Although, the corresponding time at 49°C would be more than 100 h due to irregular reduction. Hence, temperatures as low as 49°C is not to be recommended for sanitation treatments of organic waste. It is also important to note that temperatures mentioned are minimum temperatures and that higher temperatures in parts of the material cannot be used to lower the overall time for the process.

The above mentioned intervals are based on the assumption that good management practice in terms of the sanitation part of the treatment is upheld within the full-scale plants, *e.g.* the high temperature is kept

throughout the whole material. If high temperatures are not kept within the whole material the presence of P/M zones can present a risk for regrowth of pathogens (Paper I). Regrowth of pathogens can lead to lower reduction of pathogens than expected. Hence, P/M zones should be avoided or at least taken into account during sanitation treatment of organic waste, this can be achieved through the use of well insulated reactors aerated with preheated air.

Conclusions

- Growth of pathogens such as *Salm.* Typhimurium can occur in compost material at psychrophilic (<15°C) and mesophilic (15-40°C) temperatures.
- The risk of pathogen growth in compost material at psychrophilic and mesophilic temperatures is reduced when the maturity of the compost material is increased.
- The risk of pathogen growth or regrowth in compost is reduced if a high temperature is kept throughout the whole material.
- The growth of pathogenic bacteria can result in increased treatment times in order to reach the appropriate hygiene standards.
- Pasteurisation at 70°C for 60 min is sufficient to achieve a 5 $\log_{10}$ reduction in *Salm.* Senftenberg W775 and *Enterococcus* spp. and a 3 $\log_{10}$ reduction in bacteriophage ΦX174 in NaCl and in fresh and dried cattle manure under laboratory conditions. This time and temperature combinations is not sufficient to achieve a 3 $\log_{10}$ reduction in PPV.
- At both 52 and 55°C, a sufficient reduction to reach the requirements set by EC regulation for bacteria (5 $\log_{10}$) can be reached in fresh cattle manure within 16.9 h at 55°C and within 17.2 h at 52°C, but for thermoresistant viruses such as PPV, a significantly longer time is needed to reach the required reduction (3 $\log_{10}$).
- Although a reduction in microorganisms occurs at 49°C it is irregular, with rather large confidence intervals. Hence such low temperatures cannot be recommended in a sanitation treatment for materials such as ABP category 3 and manure.
- The inactivation of viruses needs to be further investigated so that possible effect of adsorption to solids in manure can be separated from the actual inactivation. This could be important when setting time and temperature requirements for reduction in a future validation process.

Future research

The time and temperature intervals for sanitation of ABP category 3 and manure have to be further investigated with the aim of validating alternative sanitation treatments. The validation of alternative time and temperature options is of great interest to the industry and it would be to prefer if simple guidelines for time and temperature combinations could be given.

As shown in this thesis, the inactivation of viruses seems to increase with decreased moisture content. However the range of conditions in which this actually occurs must be investigated further. If the perceived increased inactivation of viruses is caused by adsorption of the virus to solids, it is important to take this into consideration when validating processes for thermal inactivation of viruses.

An increased use of bacteriophages as indicators for viruses in organic waste is tempting but has to be more closely investigated. Through using bacteriophages as indicators, the expenses associated with virus analysis can be cut, with simple, fast and rather cheap analysis for bacteriophages. Furthermore, coliphages and F-RNA phages can be present in the materials in sufficiently high numbers to allow inactivation studies to be performed without adding organisms to the material.

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