Recycled Biowaste as a Source of Infection

Leena Sahlström
Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 70

ISSN 1652-6880
ISBN 91-576-7119-2

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Sahlström, L. 2006. Recycled Biowaste as a Source of Infection. Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7119-2

Biowaste and sewage sludge can be used as a fertiliser and soil amendment in agriculture. However if not treated efficiently before use, such products can contain microbial pathogens that pose a health risk for humans and animals. This study investigated the pathogen content, mainly pathogenic and antimicrobial-resistant bacteria, present in sewage sludge and biowaste. It also assessed the effects of different treatment methods on various pathogens by analysis of the pathogen content of sewage sludge and biowaste substrate before and after treatment.

Compared to sewage sludge, biowaste contained fewer pathogens, both in the untreated substrate and the digested residue. Frequent findings of *Salmonella* spp. in sewage sludge were the main reason for the difference. In addition, vancomycin resistant enterococci (VRE) were frequently isolated from sewage sludge. PFGE and PhenePlate analyses showed that both VRE and *Salmonella* spp. were capable of persisting for some months and up to two years, respectively, in the sewage sludge. Thus sewage sludge may act as a reservoir of *Salmonella* spp., VRE and other pathogens.

Pasteurisation (70°C, 60 minutes) proved to be an effective sanitation treatment for biowaste and in combination with anaerobic digestion it resulted in better inactivation of pathogens and indicator bacteria than the treatments currently used at Swedish wastewater treatment plants. In further studies at laboratory scale, pasteurisation effectively inactivated the majority of pathogens and indicator bacteria analysed except heat resistant (parvo-) viruses and spore-producing *Clostridium perfringens*.

The pathogens and antimicrobial resistant bacteria found in sewage sludge and biowaste could be spread to humans and animals if these treated wastes are used in agriculture. However, it is possible to diminish the pathogen load in the wastes so that the risks to the community are minimised. It is important to be aware of the general hygiene risks associated with the biowaste and sewage sludge and to handle, treat and use these products accordingly.

Keywords: Agriculture, anaerobic digestion, antimicrobial resistant bacteria, biowaste, *Campylobacter* spp., *E. coli* O157, recycling, *Salmonella* spp., sewage sludge, zoonoses

Author's address: Leena Sahlström, Department of Wildlife, Fish and Environment, National Veterinary Institute, SE-751 89, UPPSALA, Sweden. Leena.Sahlstrom@sva.se

Sammanfattning

För ett uthålligt kretslopp mellan stad och land kan näringsämnen i biologiskt avfall tas till vara genom att återföras till jordbruket eller miljön. Detta kan göras t.ex. genom att utnyttja slam från reningsverk eller biologiskt avfall, vilka har behandlats vid biogasanläggning, som gödning och jordförbättring i jordbruket. Avhandlingen belyser problematiken med möjliga smittrisker vid användningen av biologiskt avfall, främst avloppsslam. Smittriskerna innefattar både humana och animala sjukdomsframkallande mikroorganismer (patogener), här berörs främst bakterier, men även virus och parasiter. Många av dessa är zoonoser, dvs. de kan smitta mellan djur och människor. Flera har lång överlevnadstid i miljön och många bakterier kan dessutom föröka sig i miljön under gynnsamma förhållanden.

Den hygieniska kvaliteten i rötrest från biogasanläggningar bedömdes vara avsevärt bättre än i slam från reningsverk. Studierna innefattade analys av förekomst av patogena bakterier och antalet indikatorbakterier i obehandlat och behandlat material från anläggningarna. En påtaglig skillnad i hygienisk kvalitet var *Salmonella* spp. som frekvent isolerades från slam från svenska reningsverk. Med molekylär epidemiologiska studier (PFGE, Pulsfältsgelelectrofores) har vi kunnat härleda *Salmonella* spp. från slam tillbaka till humana sjukdomsfall. Dessutom har visats att salmonella kan leva kvar i reningsverk i upp till minst två år. Detta indikerar att slam kan utgöra en reservoar för patogener vilket medför en ökad risk vid användandet av slam, om inte adekvat hygienbehandling först utförts.

Pastörisering (70˚C i 60 min) i kombination med anaerob rötning, som används vid biogasanläggningarna, är med avseende på hygieniseringseffekten bättre än de konventionella behandlingsmetoder, som vanligen används och studerats i avloppsreningsverk. Termofil rötning har också en bättre hygieniserande effekt än mesofil rötning. Pastörisering har studerats närmare i laboratorieskala med avseende på dess reducerande inverkan på både bakteriella och virala patogener, parasiter, indikatorbakterier och bakteriofager. Pastöriseringen fungerar bra som hygieniseringsmetod men har inte tillräcklig effekt på värmetåliga (Parvo) virus och sporbildande bakterier som t. ex. *Clostridium perfringens*.

Dessutom har förekomsten av antibiotikaresistenta bakterier i slam studerats. Vancomycinresistenta enterokocker (VRE), som utgör en ökande risk genom s.k. sjukhussjuka, isolerades frekvent ur slam. VRE verkar precis som *Salmonella* spp. ha förmåga att leva kvar och eventuellt föröka sig i reningsverket. Det finns risk att antibiotikaresistenta bakterier kan spridas via slam till miljön och att resistensgenerna kan spridas mellan bakterier och eventuellt till andra bakteriearter.

Slam innehåller sammanfattningsvis både patogener och antibiotikaresistenta bakterier, som kan utgöra en risk för human- och djurhälsan, om slammet sprids utan tillräcklig hygienbehandling. Slam kan dock behandlas så att risken minimeras. En viktig del av smittskyddet är att vara medveten om smittriskerna och behandla och använda avfallet därefter.

Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Sahlström, L., Aspan, A., Bagge, E., Danielsson-Tham, M-L. & Albihn, A. (2004) Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. *Water Research* 38, 1989-1994.

II. Bagge, E., Sahlström, L. & Albihn, A. (2005) The effect of hygienic treatment on the microbial flora of biowaste at biogas plants. *Water Research* 39, 4879-4886.

III. Sahlström, L., Bagge, E., Emmoth, E., Holmqvist, A., Danielsson-Tham, M-L. & Albihn, A. (2006) A laboratory study of survival of selected microorganisms after heat treatment of biowaste used in biogas plants. Submitted.

IV. Sahlström, L., De Jong, B. & Aspan, A. (2006) *Salmonella* isolated in sewage sludge traced back to human cases of salmonellosis. *Letters in Applied Microbiology* 43, 46-52.

V. Sahlström, L., Albihn, A., Aspan, A., Rehbinder, V. & Bengtsson, B. (2006) Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. Submitted.

Abbreviations

ABP	Animal by-products
BSE	Bovine spongiform encephalopathy
EHEC	Enterohaemorrhagic *Escherichia coli*
PFGE	Pulsed field gel electrophoresis
SARS	Severe acute respiratory syndrome
SRM	Specified risk material
TSE	Transmissible spongiform encephalopathy
VRE	Vancomycin resistant enterococci
WWTP	Wastewater treatment plant

Key definitions

Biowaste: Manure and all kinds of biodegradable wastes that are separated at source in households for composting or other biological treatment purposes, as well as biodegradable waste from the whole food chain and medical factories including animal by-products (ABP). Sewage sludge is not included in the definition of biowaste.

Biogas plant: Large-scale biogas production where different kinds of biowaste including manure from livestock is used as substrate. No sewage sludge is used in Swedish biogas plants.

Pathogen: Infectious microorganism that causes illness/infection in people and/or animals.

Sewage: Wastewater entering the WWTP from households, including blackwater from flush toilets, greywater and in some cases stormwater. It may also include wastewater from businesses and industries.

Sewage sludge: The organic matter separated from sewage.

Slurry: A mixture of faeces and urine.

Wastewater treatment plant (WWTP): Formerly ‘sewage treatment plant’. Plant treating wastewater (sewage) and producing sewage sludge and treated wastewater, usually released to a nearby water recipient.

Introduction

The use of biowaste and sewage sludge on arable land will close the loop of plant nutrient recycling in a sustainable society. It will also utilise the soil amendment capacity of the biowaste and sewage sludge. However, there are many potentially harmful components for humans, animals and the environment in biowaste and sewage sludge; for example heavy metals (such as cadmium (Cd), copper (Cu) and zinc (Zn)), organic contaminants, pharmaceutical residues (such as hormones and antimicrobial agents), and pathogens. Biowaste and sewage sludge must be used in a way that is safe for humans, wildlife and domestic animals and the environment, both now and in the future. This thesis deals with the content of pathogens, in particular pathogenic and antimicrobial resistant bacteria, in sewage sludge and biowaste, and with the effects of different treatment methods on the pathogen content.

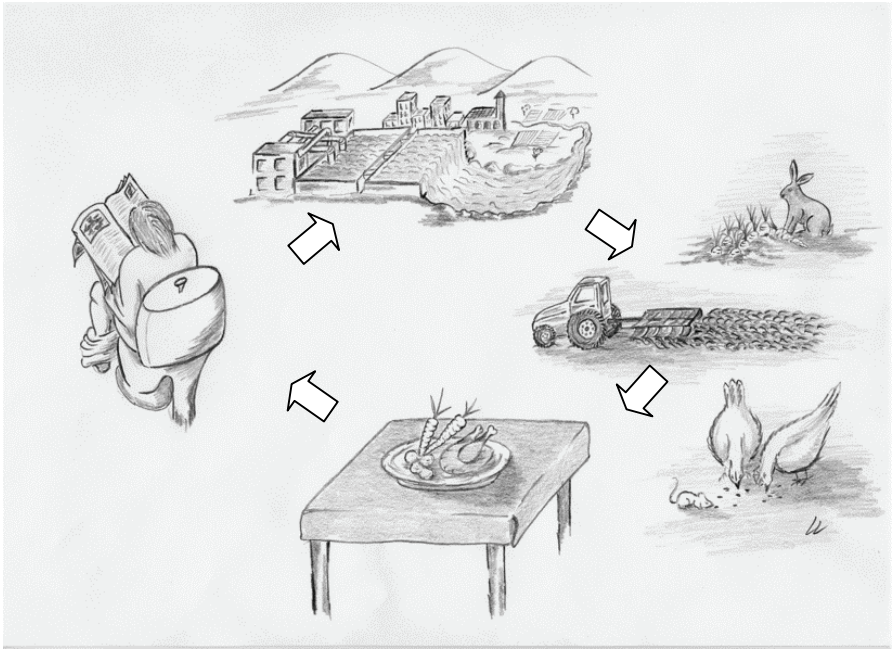

Figure 1. The use of biowaste and sewage sludge in agriculture will enable pathogens to be transferred by vector animals, contaminated water or food to humans and animals. Furthermore, infected humans will pass the pathogens back to the wastewater treatment plant, and infected animals will pass the pathogens to biogas plants as contaminated biowaste *i.e.* manure and animal by-products. Illustration: Lasse Lind.

Use of biowaste and sewage sludge

The spread of disease to humans, wildlife and domestic animals may increase if biowaste and sewage sludge are not treated efficiently before use. Agriculture has changed during the last century, with larger and more specialised farms. Farmyard

manure is no longer used exclusively on-farm but may be transported long distances, facilitating the spread of infection to other farms and regions. There is a need for treatment of large amounts of manure, especially in conjunction with disease outbreaks, to avoid the spread of infection.

Biowaste, including livestock manure, may be used as substrate in large-scale biogas plants and the methane produced may be utilised for example as fuel for vehicles or domestic heating. The digested residue is used as a soil amendment and fertiliser in agriculture.

Animal by-products are an important part of biowaste in terms of biosecurity. The use of biowaste has been restricted since May 2003 by EC Directive 1774/2002, mainly because animal by-products can be contaminated with pathogens such as prions, which cause Mad Cow Disease or other Transmissible Spongiform Encephalopathy (TSE) in ruminants. Animal by-products are divided into three different categories, as shown in Table 1: Specified risk material (Category 1) includes some specified organs that are likely to contain prions in the case of Mad Cow Disease or other TSE. Category 1 material must be treated separately and destroyed, which is mainly performed by incineration. Category 2 materials, which include manure intended for commercial purposes, require sterilisation, *i.e.* heating to at least 133°C for at least 20 minutes, at a pressure of at least 3 bar. Category 3 materials, animal by-products used *e.g.* as substrate in biogas plants, must be pasteurised (70°C for 1 hour) before further treatment (Table 1). However, there are exceptions to the treatment specifications for manure. These include pasteurisation in a biogas plant if the authorities permit this for biosecurity reasons (EC 1774/2002). The use of animal by-products at biogas plants has decreased due to the EC regulation that requires destruction of specified risk material. This is because Category 2 and 3 materials must be considered as specified risk material if they are mixed with Category 1 materials, which is a common way of handling Category 2 material *e.g.* in Sweden. In addition to the treatment requirements, there are also restrictions on the land use associated with Category 2 and 3 materials.

Table 1. Comprehensive summary of categorisation and treatment requirements of animal by-products (ABP) according to EC 1774/2002. SRM=Specified risk material, BGP = Biogas plant, TSE=Transmissible spongiform encephalopathy

ABP category	Examples of material included	Treatment requirements	Accepted use
Category 1	SRM	Destruction: Incineration, landfill or burial	None
Category 2	Organs from ruminants other than SRM, ABP from other animals than ruminants, manure[1]	Sterilisation 133°C, 20 min, 3 bar pressure[2]	Technical use, use at BGP and composting
Category 3	ABP approved for human consumption	Pasteurisation 70°C 60 min, followed by stabilisation	Animal feed[3], use at BGP and composting

[1] Intended for commercial purposes
[2] Exceptions for manure exist
[3] For some species only

Sewage sludge is not used at biogas plants in Sweden but anaerobic digestion is used as a treatment of sewage sludge at larger Swedish wastewater treatment plants (WWTP). Treated sewage sludge is used as a fertiliser and soil amendment in agriculture but nowadays in Sweden to an even larger extent at golf courses, on road verges, as a cover for landfills and mine tailings, for soil production, in private gardens and for other environmental uses (SCB, 2004). In Sweden, the use of sewage sludge on arable land is restricted by the Swedish Farmers' Union and by food producer organisations. In 2002, only 10% of sewage sludge was spread on arable land in Sweden (SCB, 2004). Furthermore, disposal of sewage sludge and biowaste on landfills (dumps) has been restricted by law since January 2005 (SFS 2001:512).

Spread of infection from biowaste and sewage sludge

An infectious agent can enter a host through the oral route, via inhaling contaminated aerosols (Carducci *et al.*, 1995), through skin lesions or through the eyes. Direct transmission occurs through direct contact with sewage sludge or biowaste, *i.e*. when persons handling the waste at *e.g*. a WWTP become infected. Due to the large amounts of infectious organisms and endotoxins in the sewage treatment environment, workers often suffer from symptoms in the gastrointestinal tract (Khuder *et al.*, 1998) and respiratory tract (Seuri *et al.*, 2005; Thorn *et al.*, 2005). During their first years of employment in particular, personnel may suffer from a higher frequency of gastroenteritis and toxic pneumonia (Thorn *et al.*, 2005).

If contaminated sewage sludge or biowaste is spread in the environment, it enables infections to be spread to animals and humans. The indirect pathway concerns a larger proportion of the human and animal population than the direct infection pathway. To be transmitted indirectly, the pathogen must reach the environment, where it must persist or possibly multiply to reach a dose that is infective (Moe, 2004). Indirect transmission of infection may occur through contamination of water (Cotruvo *et al*., 2004) or through contamination of pasture, feed or food fertilised with insufficiently treated sewage sludge or biowaste (Solomon *et al.*, 2002; Islam *et al.*, 2004). This foodborne pathway is related to recent changes in eating habits in the Western world, with increasing consumption of raw or part-cooked fruit and vegetables. Diseases can also be transmitted from contaminated land by vectors, for example insects, rodents or birds (Palmgren, 2002) that enter animal houses and homes. In addition to contamination of food, water and feed transmission may also occur through pathogens in sewage and sewage sludge that are spread on willow coppice, forest and wetlands that are used as recreational areas (Carlander, 2006). Surface water run-off or contamination of groundwater may also cause disease outbreaks (Gallay *et al.*, 2006).

Transmission of infections and disease outbreaks

The globalisation of world trade and the increased transportation of people and animals all around the world enhance the transfer of infections and diseases. The introduction of 'new' diseases such as BSE (Bovine Spongiform Encephalopathy

or Mad Cow Disease), SARS (Severe Acute Respiratory Syndrome) and avian influenza H5N1 (Bird Flu) has become a health concern world-wide.
There are few reported cases with evidence that infections in animals or people have originated from environmental sources. One probable reason for the lack of documented cases is that retrospective epidemiological studies are complicated, especially when environmental contamination is suspected. However, Reilly *et al*. (1981) reported 26 episodes of *Salmonella* in Scotland that were assumed to originate from environmental sources; land fertilised with sewage sludge was the believed reason for infection in cattle (Reilly *et al.*, 1981). Other incidents in Scotland were caused by abattoir waste being discharged into watercourses, land contaminated after flooding, seagulls transmitting the disease and run-off from fertilised land (Reilly *et al.*, 1981). Jack & Hepper (1969) reported one outbreak of *Salmonella* that was caused by cattle grazing on slurry-irrigated pasture (Jack & Hepper, 1969). Recently (2005) there was an outbreak of EHEC (Enterohaemorrhagic *Escherichia coli*) infection in Sweden where approximately 130 persons were infected during a period of several months (Anonymous, 2005). According to epidemiological studies, this outbreak was caused by lettuce irrigated with *E. coli* O157-contaminated streamwater. The same *E. coli* O157 that was isolated from the patients was also found in cattle in the same area as the lettuce fields. Solomon *et al.* (2002) demonstrated in a previous study that *E. coli* O157 is able to invade lettuce plant tissue, but it was not possible to isolate the bacteria in the Swedish outbreak from the lettuce *per se*. However, many epidemiological facts identified the irrigation of the lettuce as the cause for the Swedish outbreak (Anonymous, 2005). Another waterborne *E. coli* O157 outbreak occurred in Ontario in 2000, where six people died and several hundred people were infected through the community water supply system, which was believed to have been contaminated by run off from cattle pastures (Schroeder& Wuertz, 2003). Gallay *et al.* (2006) report an outbreak of *Campylobacter coli*, rota and norovirus caused by faecal contamination of groundwater. In Scotland, scouts camping on sheep pasture were infected with *E. coli* O157 15 weeks after the sheep were removed (Ogden *et al.*, 2002).

Microorganisms in biowaste and sewage sludge

Biowaste and sewage sludge contain pathogens of different species (Table 2) (see reviews by: Larsen &Munch; 1986; Strauch; 1991; Dumontet *et al.*, 2001). These originate from diseased animals and people, and from healthy carriers who excrete pathogens in faeces, urine and exudates. Many pathogens are zoonoses, *i.e*. they may infect both humans and animals, which enhances the spread of infections. Pathogens of importance regarding biowaste and sewage sludge include three main groups of organisms: bacteria, viruses and parasites (*i.e*. protozoa and helminths). An important factor for disease transmission through the environment that differentiates these groups from each other is their mode of replication, *i.e*. bacteria may be able to multiply in the environment independent of a host if the environmental conditions are favourable (Gibbs *et al.*, 1997). In addition, bacteria have several ways to resist environmental stress. One example of this is the spore-forming capacity among some bacteria, *e.g*. *Bacillus* spp. and *Clostridium* spp., as a response to environmental stress. For example *Bacillus anthracis* form

endospores when the organisms are exposed to oxygen (Carter & Chengappa, 1991). The vegetative bacterial cells differentiate to extremely resistant endospores that can resist desiccation, heat, acids and many disinfectants; endospores are the most resistant cell type known. Bacteria may also transfer into a state called viable but non-culturable (Oliver, 2005). Viruses and parasites need a host for replication, and are unable to multiply in the environment. However, some viruses are known to be excreted in extremely high amounts from infected individuals, up to 10^{11} viral particles per gram faeces are reported for rotavirus (Koopmans & Duizer, 2004). Viruses invade a host cell and use the replication mechanism of the host to multiply. Viruses may be rather stable in the environment and resistant to acidic conditions (pH 3-5) (Koopmans & Duizer, 2004; Vasickova *et al.*, 2005). Parasites, however, may have enhanced survival in some stages of their replication that are not dependent on a host. *Ascaris* spp. ova, for example, are very resistant to environmental conditions such as drying and cold. Whenever the ova reach a new host, they can undergo a new cycle of development and replication.

Table 2. Some examples of infectious agents found in different kinds of biowaste and sewage sludge

	Pathogen	Isolated from	References
Bacteria	*Salmonella* spp.	Anaerobically digested sewage sludge	Sahlström *et al.*, 2004 Dudley *et al.*, 1980 Jones, 1980
		Biowaste	Bagge *et al.*, 2005
	Listeria spp.	Raw sewage	Sahlström *et al.*, 2004
		Treated sewage sludge	De Luca *et al.*, 1998
		Biowaste	Bagge *et al.*, 2005
	Campylobacter coli	Composted sewage sludge	Sahlstrom *et al.*, 2004
		Primary sludge	Stampi, 1998/99
	and *jejuni*	Biowaste	Bagge *et al.*, 2005
	E. coli O157	Raw sewage	Sahlström *et al.*, 2004
		Cattle manure	Dorn, 1993
		Biowaste	Bagge *et al.*, 2005
	Mycobacterium spp.	Treated sewage sludge	Dudley *et al.*, 1980 Pickup *et al.*, 2006
	Clostridium spp.	Anaerobically digested sewage sludge	Dudley *et al.*, 1980
	Yersinia spp.	Anaerobically digested sewage sludge	Dudley *et al.*, 1980
	Shigella spp.	Sewage sludge	Dudley *et al.*, 1980
Virus	Entero	Wastewater & sewage sludge, Soil	Carducci *et al.*, 1999 Strauch, 1991
	Reo	Sewage	Carducci *et al.*, 1999
Helminths	*Ascaris* sp.	Treated sewage sludge	Gaspard *et al.*, 1995
Protozoa	*Giardia* cysts	Sewage	Fayer, 2004

Infectious dose

Actual infection is dependent not only on the pathogen species but also on the infectious dose (Table 3). Some pathogens are extremely virulent and only a few organisms are needed to make the host sick, whereas others are needed in larger amounts. Different factors may also influence the infectious dose, for example the

health status and age of the host are important factors for whether the infection will occur. There is an increasing group of immuno-suppressed people; HIV-positive and cancer patients, pregnant women, the elderly and children (Gerba *et al.,* 1996). These people are sensitive to opportunistic infections, succumb a lower dose of infectious agents and more easily acquire infections than healthy people. This is a group of people that must be taken into special consideration with respect to the spread of infection.

Table 3. Infectious dose for healthy humans of some pathogens

Microorganism	Infectious dose	References
Salmonella spp.	10^4 bacteria	D'Aoust *et al*., 2001
C. jejuni.	<1000 bacteria	Nachamkin, 2001
L. monocytogenes	>100 bacteria/g*	Swaminathan, 2001
E. coli O157	4-20 (<50) CFU	Strachan *et al.*, 2001
Y. enterocolitica	$>10^4$ CFU	Robins-Browne, 2001
Virus	1-10 virus particles	Vasickova *et al.*, 2005
Giardia lamblia	10-100 cysts	Smith & Grimason, 2003

*Number in contaminated food responsible for foodborne human cases

Examples of pathogens in biowaste and sewage sludge

The bacterial pathogens and parasites in biowaste and sewage sludge discussed below are zoonotic, as well as the viruses SARS, avian influenza, hepatitis E and probably norovirus.

Salmonella spp.

Salmonella is one of the most likely pathogens to be spread in the environment by animal slurry, manure and sewage sludge (Jones, 1980). *Salmonella* spp. are Gram-negative, rod-shaped, motile bacteria and belong to the family *Enterobacteriaceae* (Carter & Chengappa, 1991). All serovars of *Salmonella* spp. are potentially pathogenic to both animals and people. However, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S*. Enteritidis) is the most common among the reported human S*almonella* infections in Sweden, followed by *S.* Typhimurium and *S.* Hadar (Sahlström *et al*., 2006). Foodborne enteritis or so called food-poisoning, including symptoms of diarrhoea, nausea and stomach-ache, is the most common infection caused by *Salmonella* spp., but *Salmonella* spp. are also associated with waterborne infections. Persons infected with *S.* Typhi (typhoid fever) may in 5% of cases continue to shed bacteria up to 1 year after recovery from the disease (Schroeder & Wuertz, 2003). *Salmonella* spp. may grow in temperatures ranging from 6°C to 47°C and survive in slurry for more than 77 days (Mitscherlich & Marth, 1984). A survey in Norway has revealed that 10% of sewage sludge samples from Norwegian WWTPs were salmonella-positive (Rosef, 1999). In Denmark, sewage sludge is considered salmonella-positive if the WWTP serves more than 4 000 people (Larsen, 1995). There is a low frequency of salmonella-infected animals and domestic human *Salmonella* infections in Sweden

due to a strict *Salmonella* control programme in livestock, and therefore Sweden is eager to prevent the spread of infection among livestock (Anonymous, 2001).

Campylobacter spp.

Campylobacter spp. are Gram-negative microaerophiles, spirally curved rod-shaped bacteria and they are motile by a single polar flagellum (Carter & Chengappa, 1991). They are the most common cause of waterborne bacterial gastroenteritis throughout the world (Schroeder & Wuertz, 2003). *Campylobacter* are often connected with outbreaks caused by faecal contamination of water (Gallay *et al.*, 2006). *Campylobacter* are likely to be transported significant distances from the disposal site by underground waters (Stampi *et al.*, 1998/99). *Campylobacter* is also a cause of food poisoning, commonly through raw or insufficiently cooked poultry (Berndtson, 1996). In many countries, *e.g.* Sweden, *Campylobacter* spp. (together with *Salmonella* spp.) are one of the major bacterial reasons of gastroenteritis in humans. *Campylobacter* spp. cause diarrhoea, nausea, vomiting, fever, abdominal cramping and chills. The incubation period of campylobacteriosis is 24 h, it is usually self-limiting and the duration of symptoms is on average 3 days (Berndtson, 1996). *Campylobacter jejuni* is the cause of 90% of human *Campylobacter* cases. Other thermophilic *Campylobacter* spp. are *C. coli, C. lari* and *C. upsaliensis*. *Campylobacter jejuni* has been found in sludge (Stampi *et al.*, 1998/99: Steltzer *et al.*, 1991). *Campylobacter* spp. are quite sensitive to mesophilic anaerobic digestion and are not considered a major problem in anaerobically digested sludge (Stampi *et al.*, 1998/99; Steltzer *et al.*, 1991). However, Kearney *et al.* (1993) showed in an investigation of mesophilic anaerobic digestion of slurry under laboratory conditions that viable organisms of *C. jejuni* could still be detected when stored for 112 days after digestion.

EHEC (Enterohaemorrhagic Escherichia coli)

E. coli O157 are Gram-negative, rod-shaped and belong to the family *Enterobacteriaceae*. During recent years, verotoxin-producing *Escherichia coli* O157 (and other serotypes) has become an important waterborne and foodborne pathogen that causes enteric disease, including bloody diarrhoea and abdominal cramps. A serious complication is the life-threatening haemolytic uremic syndrome (HUS) causing renal failure, which mainly affects children and elderly. The verotoxins produced inhibit protein synthesis in eukaryotic cells and cause damage in endothelial cells, which play a role in haemorrhagic colitis and haemolytic uremic syndrome. An EHEC infection may be fatal. Cattle and other ruminants are the main reservoir of *E. coli* O157 (Dorn, 1993; Kudva *et al.*, 1998; Molbak & Scheutz, 2004). In Sweden, 10% of cattle herds are infected and almost 23% of the herds in areas in the south-west of Sweden carry *E. coli* O157. The bacteria are found in bovine manure, which is a common substrate in biogas plants and hence a possible source of contamination of the environment. Wang *et al.* (1996) report that *E. coli* O157:H7 survives and can produce verotoxins for up to 10 weeks in manure. Wang *et al*. (1996) even demonstrate that *E. coli* O157:H7 is able to multiply in bovine faeces at 22 and 37°C.

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive, motile rod (Carter & Chengappa, 1991), which can survive and even grow at 1°C-45°C (Junttila *et al.*, 1988). *L. monocytogenes* is ubiquitous and found in soil, silage, water and decaying vegetation (Bille, 1996). For humans, *L. monocytogenes* is primarily considered a foodborne pathogen. It may cause different clinical manifestations such as flu-like symptoms in healthy persons, and spontaneous abortion in both ruminants and humans. De Luca *et al*. (1998) demonstrated that sewage sludge contains *L. monocytogenes*. This makes *L. monocytogenes* a pathogen that should be considered as a potential health risk in recirculation of biowaste and sewage sludge.

Mycobacterium spp.

Mycobacterium spp. are rod-shaped, aerobic, non-motile, acid-fast bacteria which are highly resistant to various environmental conditions (Hirsh & Zee, 1999). *Mycobacterium tuberculosis* is the main causal agent of tuberculosis in humans. However, *M. bovis,* the main cause of tuberculosis in animals, may also cause disease in humans. Tuberculosis is mainly spread by aerosols but oral transmission occurs as well. Cattle are considered free from *M. bovis* in Sweden (http://www.sva.se, 9-Aug-2006). However, there is a low prevalence of tuberculosis in Sweden; approximately 400 tuberculosis (majority *M. tuberculosis* and a few *M. bovis*) cases among humans are reported each year (Anonymous, 2006). These are either persons infected early in life when tuberculosis was common, or immigrants from countries with tuberculosis. Humans with manifest renal *M. bovis* may excrete the bacteria in their urine, which may possess a threat in the use of urine as fertiliser (Grange &Yates, 1994).

Mycobacterium avium subsp. *paratuberculosis* (*M. paratuberculosis*) is found world-wide and causes severe chronic enteritis in ruminants (Hirsh & Zee, 1999). However, Swedish livestock are considered free from *M. paratuberculosis* (Sternberg & Viske, 2003). *M. paratuberculosis* are excreted in faeces of infected animals and mainly spread between animals by contaminated water or feed. There are few reports of *Mycobacterium* spp. in sewage sludge. However, Dudley *et al*. (1980) isolated unspecified *Mycobacterium* spp. from sewage sludge in Texas, USA (Dudley *et al.*, 1980) and *M. paratuberculosis* has recently been found in sludge in the United Kingdom (Pickup *et al.*, 2006).

Bacterial spore producers

Bacterial spore producers are the most resistant among bacteria. The spores can persist for decades in soil (*e.g*. *Bacillus anthracis*) and they are very persistent to heat (Mitscherlich & Marth, 1984). *Bacillus anthracis*, which causes anthrax, is Gram-positive, aerobic or facultatively anaerobic and found widely in the soil, air and water (Carter & Chengappa, 1991). *Clostridium* spp. are Gram-positive anaerobic rods and found in all kinds of environments. *Clostridium perfringens* is commonly found in the intestines of humans and animals and may under certain circumstances be pathogenic. Several severe diseases are caused by *Clostridium*

spp., such as tetanus (*Cl. tetani*), botulism, (*Cl. botulinum*) and black leg (*Cl. chauvoei*) (Hirsh &Marth, 1999). *Clostridium* spp. are excreted in faeces of infected animals and must be considered in treatment of manure on farms and at biogas plants. *Clostridium tyrobutyricum* causes production problems for cheese producers, resulting in late blowing of cheese and causing serious economic losses (Dasgupta & Hull 1989). Using digested residue as a fertiliser could contaminate the silage, because neither anaerobic digestion nor ensiling inactivate *Cl. Tyrobutyricum* (Johansson *et al.*, 2005). Thus, silage may be a source of the spores for cows and subsequently for their milk.

Other bacteria to be considered

Yersinia spp. are Gram-negative, rod-shaped facultative anaerobic bacteria commonly associated with water and are of primary concern in sewage sludge (Bitton, 1999). *Yersinia* spp. may grow in temperatures approaching 0°C (Holt *et al.*, 1994) and can cause acute enteritis, mainly in children. Pigs are an important source of *Y. enterocolitica* (Fredriksson-Ahomaa *et al.*, 2006) and a potential risk factor when animal by-products are used as substrate in biogas plants.

Shigella spp. are Gram-negative, non-motile, rod-shaped bacteria and shigellosis is mainly a disease of humans and other primates. *Shigella dysenteriae* symptoms include severe and bloody diarrhoea. It is excreted in faeces and usually transmitted by direct contact or food-poisoning, but waterborne outbreaks due to faecal contamination of water also occur (Schroeder & Wuertz, 2003).

Parasites

Protozoa and helminths infect through the faecal-oral route. Contaminated water is a common mode of transmission. In Sweden, parasites (protozoa and helminths) are not considered a major problem and are of greater concern as regards developing countries, where clean drinking water is scarce and adequate sanitation is lacking. Parasites are mainly a problem among children but also among immuno-suppressed individuals.

Giardia lamblia is a zoonose and the most common protozoan cause of gastrointestinal disease world-wide. It causes diarrhoea and is most common in children in developing countries. Giardiasis may be asymptomatic but infectious cysts are excreted in the faeces (Fayer, 2004). The infection occurs by the faecal-oral route, either by direct contact or through contaminated water or food.

Cryptosporidium causes a self-limiting diarrhoeal disease but the intensity and duration depend on the immunological status of the patient and may persist for more than one month in immuno-suppressed individuals (Bitton, 1999). *Cryptosporidium parvum* is besides cattle reported in many other mammalian species and is the second most common causal agent (after *C. hominis*) of cryptosporidiosis in humans (Bitton, 1999; Fayer, 2004).

Ascaris suum is a common intestinal helminth in swine but may occasionally infect humans (Endo & Morishima, 2004). It resembles *Ascaris lumbricoides*, which infects humans (Feachem, 1983). *Ascaris* ova may persist in the

environment for many years (Urquhart *et al.*, 1986), but seems rather sensitive to heat treatment exceeding 50°C (Endo & Morishima, 2004).

Viruses

Viruses are the most common cause of gastrointestinal infections transmitted by food world-wide (Heritage, 2003; Vasickova *et al.*, 2005). Different enteric viruses are frequently present in sludge and manure (Strauch, 1991; Dumontet *et al.*, 1999). Many viruses are resistant to environmental conditions; parvo- and circoviruses for example are very resistant to heat (Emmoth *et al.*, 2004) and enteroviruses can survive several weeks in the environment (Vasickova *et al.,* 2005). Noroviruses survive heat treatment for 30 minutes at 60°C and are also resistant to low pH (Koopmans & Duizer, 2004).

Norovirus is the cause of winter vomiting disease, with symptoms of vomiting and diarrhoea. It is transmitted mainly by airborne aerosols, but also via contaminated water, food and the environment. A Japanese study indicates that the norovirus concerned may be zoonotic, since it was found in faeces from pigs (Sugieda *et al.*, 1998).

Rotavirus, on the other hand, is the most common cause of diarrhoea in children below 3 years of age (Vasickova *et al.*, 2005). Hepatitis A virus, together with norovirus, is the most common food-borne pathogen (Koopmans & Duizer, 2004). Hepatitis E virus has been detected in both pigs and humans and is now considered zoonotic (Meng *et al.*, 1997; Koopmans & Duizer, 2004), but the zoonotic transmission of Hepatitis E is believed to be rather low. Hepatitis E virus has been involved in waterborne outbreaks (Koopmans & Duizer, 2004).

Foot and Mouth Disease is caused by a very contagious virus that infects cloven-footed animals (http://www.oie.int, 8-Aug-2006) The importance of Foot and Mouth Disease is mainly the production losses and the rapid airborne spread by aerosols. Swine Vesicular Disease is a virus infection affecting pigs that resembles Foot and Mouth Disease (House & House, 1999) and that is why this virus, like Foot and Mouth Disease, is also on the OIE-list (Office International des Epizooties, World Organisation of Animal Health) of notifiable epizootic diseases and is compulsorily treated in the event of an outbreak (http://www.oie.int/eng/maladies/en_classification2005.htm, 8-Aug-2006).

SARS (Severe Acute Respiratory Syndrome) is believed to have been spread among humans by leakage in sewage pipes in the Amoi Gardens in Hong Kong, where there was a large SARS outbreak in 2003 (Moe, 2004).

Avian influenza H5N1 (Bird Flu) has been isolated from contaminated pond water where diseased birds have swum. It is assumed that influenza virus may be excreted in faeces from infected humans (WHO, 2006) and in the event of a pandemic this would be a risk factor in sewage sludge.

Prions

Prions were not included in the studies reported in this thesis but according to the literature, they are very heat resistant and could resist pasteurisation (70°C for 1h).

Mad Cow Disease or BSE (Bovine Spongiform Encephalopathy) is caused by a prion, which is zoonotic. In humans, the same disease is called variant-CJD (Creutzfeldt-Jacobs Disease). According to current knowledge, prions are only present in nervous and lymphatic tissue from adult animals and these tissues are classified as specified risk material. Prions are associated with meat and bone meal and not considered an issue in sewage sludge. According to EC Directive 1774/2002 concerning animal by-products, specified risk material must be treated separately and destroyed (Table 1).

Spread of antimicrobial resistance

There is an increasing problem with antimicrobial resistance in both human and veterinary medicine. Resistance among bacteria is becoming more common, but new anti-microbial drugs are not (Cookson, 2005). Resistance against antimicrobials may cause severe problems in human and animal healthcare. An example is human nosocomial infections, which can be difficult to treat due to antimicrobial resistance.

Antimicrobial resistant bacteria are clonally spread but a further dimension to the growing problem is horizontal gene transfer, where resistance genes may be transferred between bacteria of the same species or to other bacterial species or genera (Klare *et al.*, 2003). Bacteria can become resistant either by spontaneous mutation(s) or by acquisition of resistance genes from other bacteria via different mechanisms of transmission: conjugation, transformation and transduction. Conjugation (transfer of plasmids or transferable genetic elements (transposons) in conjunction with bacterial mating) is the transfer mechanism commonly shown to occur in enterococci (Klare *et al.*, 2003). Transformation implies uptake of naked DNA, while transduction involves transfer of genetic material via bacterophages.

Antimicrobial resistant bacteria are selected by antimicrobial pressure such as use of antibiotics in human and veterinary medicine (Andersson, 2003) or through the presence of antibiotics in a WWTP (Guardabassi *et al.*, 2002). Resistance genes have been considered a burden for the bacteria and in absence of antibiotic pressure, the resistance genes could be lost (Andersson, 2003). On the other hand, some researchers suggest that the resistance genes can be carried regardless of antibiotic pressure (Andersson, 2003).

Vancomycin resistance

Antimicrobial usage in animal husbandry and human medicine in Sweden has been low compared to that in other countries (Cars *et al.*, 2001). Antimicrobial feed additives are not used as growth promoters in Sweden. The use of avoparcin, an analogue to vancomycin, which was previously used as a growth promoter in animal husbandry and is the believed cause of the common occurrence of vancomycin resistant enterococci (VRE) in European livestock, was prohibited in Sweden in the early 1980s. Despite this, there is still a rather high frequency of VRE in Swedish sewage (Iversen *et al.*, 2002). Because of the way WWTPs work, using bacterial adhesion to particles in their treatment process (Godfree & Farrell, 2005), it is expected that VRE are found in sewage sludge as well.

Transfer of antimicrobial resistance between bacteria has been shown to occur in WWTPs (Mach & Grimes, 1982; Marcinek *et al.*, 1998). This may increase the amount of bacteria resistant to antimicrobials in sewage sludge.

High level vancomycin resistance, coded by *vanA*, is the most common vancomycin resistance in enterococci causing nosocomial infections in humans and the only vancomycin resistant trait that has so far disseminated to *Staphylococcus aureus* (Courvalin, 2005). The *vanA* gene is usually located on transposon Tn*1546*. Moderate level resistance against glycopeptides, coded by *vanB*, is commonly located on transposon Tn*1547*. Bacteria may be intrinsically resistant against glycopeptids such as the *vanC* resistance in some *Enterococcus* species, *e.g*. *E. gallinarum*. This intrinsic resistance is low level, chromosome located and not transferable (Courvalin, 2005).

Treatment options for biowaste and sewage sludge

In the early history of WWTPs, they were mainly built to prevent the transmission of diseases caused by poor sanitation systems (Höglund, 2001). Today, however, WWTPs focus on many other important aspects of cleaning wastewater besides reducing the microbial pollutants. Mechanical, biological and chemical treatment of sewage is carried out, mainly to reduce organic matter, phosphorus and nitrogen. Pathogens adhere to the sludge particles and consequently the most concentrated infectious load is in the sewage sludge compared to the sewage (Godfree & Farrell, 2005).

Biological treatment of biowaste and sewage sludge aims at stabilising the sludge so that the volume of sludge, the amount of organic compounds and the odour is decreased. The stabilisation further decreases the attractiveness for potential vector animals. Stabilisation is not optimised for reduction of pathogens, but sanitation may also occur to various degrees (Godfree & Farrell, 2005). Incineration is one effective way of killing pathogens, but in this way one loses the soil amendment capacity of the biowaste. Biological methods, such as anaerobic digestion and composting, are common ways of stabilising biowaste and sewage sludge. WWTPs use different types of treatments to stabilise the sludge and depending on the method, there is a greater or lesser reduction in pathogens (Godfree & Farrell, 2005).

Factors affecting inactivation of pathogens

The decrease in microorganisms in treatment of biowaste and sewage sludge is dependent on many different environmental factors (Carrington, 2001; Höglund, 2001; Sahlström, 2003) such as temperature, time, available nutrients, pH, moisture and radiation (Godfree & Farrell, 2005). Inactivation of pathogens is also dependent on species, as they are more or less sensitive to external conditions. Several of the relevant microorganisms are resistant to environmental factors and very persistent in the environment.

Temperature and its correlation with time are the most important factors for reducing pathogens in treatment of biowaste and sewage sludge (Sahlström,

2003). A shorter time is needed for inactivation of pathogens at higher temperatures. Competition among microorganisms for nutrients is a limiting factor that reduces pathogen amounts in *e.g.* mesophilic anaerobic digestion (Smith *et al.*, 2005).

Traditional chemical treatments performed with lime (slaked lime or quicklime) are mainly based on extreme changes in pH. Inactivation of pathogens using slaked lime ($Ca(OH)_2$) treatment relies on an increase in pH to >12. Treatment with quicklime (CaO) simultaneously increases the temperature to 55°C (Strauch, 1991). Recently, treatment with urea that is degraded to ammonia (NH_3), which inactivate pathogens, has been proven to be an efficient sanitation method for faecal matter and manure (Vinnerås *et al.*, 2003).

Moisture and drought are additional important factors in the life and growth of microorganisms. Spore-forming bacteria have developed the ability to form spores, which are extremely resistant to drought and other environmental factors. Parasites such as *Ascaris* ova are also very drought-tolerant. UV- radiation is another factor that inactivate some microorganisms (Koivunen & Heinonen-Tanski, 2005).

Pasteurisation

Pasteurisation is an effective treatment for decreasing pathogens in biowaste (Carrington, 2001; Godfree & Farrell, 2005; Skiadas *et al.*, 2005). However, pasteurisation is not used for sewage sludge in Sweden today. In support of pasteurisation as a pre-treatment to thermophilic anaerobic digestion, (Skiadas *et al.*, 2005) reported that it not only results in better sanitisation of sewage sludge, but also enhances removal of organic matter in the subsequent anaerobic digestion and produces a higher biogas yield. Today there are 15 full-scale biogas plants in Sweden, treating mainly manure, animal by-products, residues from food industries and organic household waste. In these biogas plants, pasteurisation is used before stabilisation with anaerobic digestion. Post-pasteurisation would be more cost effective than pre-pasteurisation. However, in the early 1980s there were problems with re-contamination and growth of pathogens in the pasteurised substrate, which led to pasteurisation after digestion being abandoned (Clements, 1983; Keller, 1983). Following this a stabilisation process after pasteurisation was required.

Anaerobic digestion

Under mesophilic (approx. 30-38°C) or thermophilic (approx. 50-55°C) conditions, different anaerobic microorganisms ferment organic matter, producing energy in the form of methane (CH_4). Through a series of complex reactions, bacteria digest organic material to inorganic molecules, methane and carbon dioxide (Ad-Nett, 2000). The biogas (methane) produced can be used as energy, for instance processed to biofuel for vehicles or for domestic heating. The digested residue can be used as a soil amendment and fertiliser. In Sweden, the large-scale biogas plants that process biowaste including manure (although not sewage

sludge) for methane production use the digested residue as a fertiliser on arable land.

WWTPs also use anaerobic digestion in their stabilisation of sewage sludge. However, this is done without a preceding pasteurisation and usually under mesophilic conditions, mostly because it is cheaper and the process has a higher robustness (Skiadas *et al.*, 2005). The mesophilic anaerobic digestion is run at temperatures (30-38°C) that are close to human and animal body temperature. This means that the temperature is close to optimal growth temperature for many pathogenic bacteria and the possibility that bacteria may multiply in the digester cannot be out ruled. The reducing factor for pathogens in the mesophilic digestion process is the competition for nutrients with other microorganisms. Anaerobic digestion may be performed either continuously or batch-wise. In order to reach the best inactivation effect of pathogens, it is important to have continuous mixing of the material in the digestor, to avoid by-pass streams and to ensure that all material is stabilised (Smith *et al.*, 2005). Thermophilic anaerobic digestion gives a better hygiene quality of sewage sludge than mesophilic anaerobic digestion (Olsen &Larsen, 1987). The main pathogen-reducing factor during thermophilic anaerobic digestion is temperature in relation to time (Smith *et al.*, 2005). However, there are only a few larger WWTPs working at thermophilic temperatures in Sweden today, despite the fact that the high initial economic investment and the more expensive maintenance and running costs are rewarded with a hygienically better sludge, a larger treatment capacity due to a shorter digestion time and usually a higher yield of biogas (Zabranska *et al.*, 2002).

Composting

In windrow composting, sewage sludge is mixed with a bulking agent such as wood chips, peat or horse manure. Windrow compost is aerated by turning the material or by forced aeration (Strauch, 1991). This aeration of the material enhances the microbial activity of aerobic bacteria, which in turn increases the temperature to more than 50°C, which enhances the pathogen inactivation effect. According to UK Code of Practice for sanitation of sewage sludge in windrow composts, the temperature must reach 55°C for a minimum of four hours and 40°C for at least 5 consecutive days. Under these conditions, most bacterial and viral pathogens and parasite eggs are effectively inactivated (Godfree, 2003) However, in windrow composting it can be difficult to meet the regulations in the whole batch. With too few mixings, the temperature will not rise as expected in the whole batch and there may be areas, such as the colder surface and pockets in the batch, that do not reach the required temperature or maintain it long enough to inactivate pathogens.

Storage

Storage is a very attractive type of treatment, especially for small WWTPs, mainly because it has advantages in low investment and running costs and it is an easy way to handle sewage sludge. However, storage alone is not regarded as an effective way to inactivate pathogens in sludge (Carrington, 2001). In Sweden

sludge is stored usually for six months, in heaps outdoors on the ground or on a concrete surface, before being used.

Storage as a form of hygiene treatment has been studied in a laboratory and full-scale pilot study under Swedish (Uppsala) climate conditions for one year (Berggren *et al.*, 2004, 2005). The hygienic effect of storage of sewage sludge was evaluated through enumeration of *Salmonella* spp. and indicator bacteria, including enterococci and coliform bacteria. After two months of storage of sewage sludge, no *Salmonella* spp. could be isolated in the heaped material. However, after one year there were still >3.2 $\log_{10}$ CFU/g enterococci present in the stored sludge (Berggren *et al.*, 2005). This would imply that there could still also be pathogens present in the sludge. In a parallel laboratory study, *Ascaris* ova were analysed in sewage sludge stored at temperatures of 7, 13 and 21°C for 214 days in climate chambers (Berggren *et al.*, 2004). *Ascaris* ova were still viable at the end of the study period at all three temperatures studied. Avery *et al*. (2005) monitored the decline in *E. coli* O157 in different wastes over 64 days and concluded that storage decreases the amount of *E. coli* O157 but does not eliminate the pathogen. In an Australian study of stored sewage sludge (Gibbs *et al.*, 1997), *Salmonella* spp. were re-isolated after one year of storage, and indicator bacteria were isolated in higher amounts at the end of the study than at the beginning, which indicates bacterial growth in the stored material. In addition to re-growth of bacterial pathogens, there is also a risk for re-contamination of stored material by *e.g*. vector animals.

Evaluation of the hygiene quality of biowaste

Efficient hygiene quality is reached when a product is not infectious for healthy or immuno-suppressed people or animals. The EU has defined effective hygiene treatment such that no *Salmonella* spp. are detectable and enterococci are reduced by 3 $\log_{10}$ units (99.9%) (EC 1774/2002).

One way of measuring and evaluating the quality of biowaste and sewage sludge is to analyse the content of pathogens and/or indicator organisms present (product control). Pathogens commonly used in hygiene investigations are different *Salmonella* spp. The effect of treatment can be measured by comparing the content of the microorganism in the untreated and treated substrate. The reduction of pathogens and hygiene quality can also be assessed by monitoring the treatment process through several different checkpoints measuring the temperature, time, pH or other critical factors (process control). The process control is usually combined with spot-testing of the end-product in full-scale treatment plants.

Indicator organisms

Some pathogens are too difficult or expensive to analyse, or too dangerous to handle. Instead, various indicator organisms of faecal origin that are not harmful for humans and animals are analysed. These surrogates should be safe and easy to handle, be present in rather large quantities and cheap to analyse (Bitton, 1999). Commonly used indicators are *Enterococcus* spp., *E. coli*, coliforms, *Ascaris suum*

and various phages. The indicators are then used as model organisms for the occurrence, survival or inactivation of pathogens. Unfortunately no universal indicator microorganism has yet been found (Harwood *et al.*, 2005). However, there are several microorganisms that are used as indicators for various reasons, as presented below.

Indicator bacteria

Indicator bacteria of faecal origin commonly used for evaluation of faecal contamination are: Enterococci, *E. coli*, coliforms and thermotolerant coliforms, and *Clostridium perfringens*. Coliforms and thermotolerant coliforms are widely used as indicators for faecal contamination; however, coliforms are not exclusively of faecal origin. Enterococci are more resistant to environmental stress than coliforms, and have been suggested as an indicator for faecal contamination in water and for enteric viruses in sludge (Bitton, 1999). However, bacterial indicators are not consistently correlated with contamination of viral pathogens (Koopmans &Duizer, 2004). Enterococci are suggested as the best indicator bacteria for validation of treatment in biogas plants (Larsen *et al.*, 1994) and are used in Denmark as an indicator for *Salmonella* spp., *L. monocytogenes*, *Campylobacter* spp. and *Yersinia* spp. (Espensen, 1996). However, enterococci have limitations when the temperature exceeds 55°C, whereupon they are quickly reduced and impossible to quantify (Bendixen & Ammendrup, 1992). *E. coli* has been recommended as a reliable indicator for *S.* Typhimurium contamination in manure-fertilised soil (Natvig *et al.*, 2002). *Clostridium perfringens*, an anaerobic spore producer, is considered too resistant to act as an indicator organism for faecal contamination (Bitton, 1999).

Parasites

Ascaris suum ova are used as an indicator for parasites as they are assumed to act in a similar way to the human pathogen *Ascaris lumbricoides*. Both are highly resistant to environmental conditions (Feachem, 1983).

Bacteriophages

Viruses are generally more resistant but more difficult to analyse in environmental samples than bacteria. In contrast to viruses, bacteriophages (viruses of bacteria) are very rewarding to study, because they are not harmful to man or animals, and they are often easier and cheaper to analyse than enteric viruses. Bacteriophages are suggested as indicators for faecal pollution of enteric viruses in different water environments (Bitton, 1999). Coliphages ϕx174 (single-stranded DNA) and MS2 (single-stranded RNA) are the most commonly used phages in environmental studies (Leclerc *et al.*, 2000). Coliphages are naturally occurring in the environment and are not specific to *E. coli*, but may multiply in other Enterobacteriace species that are not correlated to faecal contamination (Leclerc *et al.*, 2000). In a study of aerosols in WWTPs, no correlation was found between the coliphage and enterovirus contents (Carducci *et al.*, 1999). *Salmonella* phage 28B has double-stranded DNA (Lilleengen, 1948). *Salmonella* phage 28B does not occur naturally in neither environmental samples nor faeces, but is suitable as an indicator in laboratory experimental settings, or in controlled environmental

studies. It has been used in water leakage trace studies (Johansson *et al.*, 1998; Carlander *et al*. 2000) and has also been used as a process evaluator for liquid composting (Eller, 1995).

Objectives

The overall aim of this thesis was to study the possibility of recycling biowaste and sewage sludge in ways that are safe with respect to spreading infections to humans and animals.

Specific objectives were to analyse the occurrence and persistence of bacterial pathogens in biowaste and sewage sludge (Papers I and II) and their survival after different treatments (Papers I, II and III), in order to evaluate possible health risks with the use of organic waste on arable land, especially sewage sludge and digested residue from biogas plants. In addition, the occurrence of antibiotic resistance in bacteria (*Salmonella* spp. and *Enterococcus* spp.) isolated from sewage sludge was studied (Papers IV and V), and the association between *Salmonella* strains found in sewage sludge and human salmonellosis was investigated (Paper IV).

Materials and methods

Materials and methods used for the specific investigations in Papers I-V are described in detail in the respective papers.

Survey of the hygiene quality of sewage sludge and digested residue from biogas plants

In order to assess the bacterial pathogen content and the effect of various conventional treatments on pathogen reduction in sewage sludge and biowaste, substrates from eight WWTPs and four biogas plants throughout Sweden were investigated for 1-2 years (Papers I and II). The eight WWTPs represented a variety of conventional treatments: mesophilic and thermophilic anaerobic digestion, sedimentation and open windrow composting (Paper I). The four biogas plants used were operating commercially in Sweden under national regulations on animal by-products at that time (2000) and using animal by-products from slaughterhouses, residues from food industries and biodegradable household waste as substrate in addition to manure. The treatment at the biogas plants consisted of pasteurisation (70°C for 60 min) combined with mesophilic or thermophilic anaerobic digestion (Paper II).

The effect of pasteurisation was further investigated under laboratory conditions. The substrate analysed in the experiment (Paper III) was untreated substrate from one of the biogas plants above. The laboratory setting was planned so as to mimic the conditions in a full-scale biogas plant in terms of heat treatment. The substrate was heated in a water bath with continuous mixing with a magnet in 250-mL bottles. Samples were analysed 30 and 60 minutes after the temperature reached 55°C and 70°C, respectively. During the laboratory scale pasteurisation study, the target temperature was reached in the whole substrate after up to 17 minutes, but during the heating the temperature varied greatly in the substrate.

In the surveys (Papers I and II) and the laboratory study (Paper III), samples of treated and untreated sewage sludge and biowaste and digested residue were analysed qualitatively with respect to pathogens (*Salmonella* spp., *L. monocytogenes*, *E. coli* O157 and *C. jejuni* and *coli*) and indicator bacteria (*Enterococcus* spp., coliform and thermotolerant coliforms, presumptive *E. coli* and the spore producer *Cl. perfringens*) were enumerated to assess the hygiene quality. In addition, *Bacillus cereus* was enumerated in Paper II. In the laboratory study on the effect of pasteurisation of substrate in biogas plants (Paper III), the pathogenic bacteria used were laboratory strains (National Veterinary Institute, SVA, Uppsala, Sweden) of the above-mentioned pathogens. In addition, three representatives of viruses were used in this study, namely porcine parvovirus, Swine Vesicular Disease virus and *Salmonella* phage 28B. Parasites were represented by *Ascaris suum* ova (Paper III). All the analyses of bacteria, parasites and virus are described or referred to in the separate papers. The analyses used were chosen to be suitable for use in routine analysis of similar substrates during continuous product control in WWTPs and biogas plants. The analysis of the virus in Paper III was adapted somewhat and evaluated to suit the specific substrate used in biogas plants.

Some of the results regarding the composted sewage sludge samples in Paper I have not been shown or discussed earlier and are therefore presented below (Table 6). These include analysis of samplings from windrow composts at a WWTP (treatment plant G in Paper I), where the sewage sludge was mixed with wood chips and composted for 3-4 weeks. The maturation took up to 6 months depending on the season and the composted material was mixed with sand before use. The personnel working at the plants were visited and instructed to take the samples approximately 30 cm deep from material in the compost heap that was stabilised and ready for use.

Antimicrobial susceptibility

Antimicrobial susceptibility in *Salmonella* spp. (Paper IV) and *Enterococcus* spp. (Paper V) was analysed by a microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) using VetMIC^{TM} panels (SVA, Uppsala, Sweden). The analysis was performed in 96-well microtitre plates, which contained doubling dilutions of the antimicrobials to be tested. The MIC (Minimum Inhibitory Concentration) is the lowest concentration of antimicrobial

agent that inhibits visible bacterial growth. In Paper IV, Salmonella isolates were classified as resistant or susceptible according to the microbiological cut-off values used in the Swedish Veterinary Antimicrobial Resistance Monitoring Programme (SVARM, 2005). Briefly, microbiological cut-off values are based on the MIC-wild-type distributions and are set to separate inherently susceptible strains from strains with acquired resistance as outlined by EUCAST (http://www.srga.org/Eucastwt/bpsetting.htm, 8-Aug-2006).

Pulsed field gel electrophoresis (PFGE)

Bacterial cells are harvested and mixed with agar and embedded in agar plugs. Chemical lysis of the bacterial cells frees the DNA. The DNA is then cleaved with restriction enzyme, which works as a scissor that cuts the DNA exactly at the same recognition site, which is specific for the respective restriction enzyme. Thereafter, the DNA fragments are separated by PFGE, which (due to its changing electric field) enables larger fragments (up to 10 Mb) to migrate in the gel matrix (Schwartz *et al.*, 1983). The PFGE patterns are then evaluated by ocular inspection or more commonly by Gelcompare software or other data analysis.

PFGE is a discriminatory genotyping method but has the disadvantages of being time-consuming and rather expensive. The rate of discrimination is even higher if more than one restriction enzyme is used and criteria for evaluation of the PFGE patterns can be set more or less strictly. Tenover *et al*. (1995) presented a model for interpretation of the PFGE patterns that is widely used. We used three different restriction enzymes and the criteria that were set and followed in the comparison of salmonella from sewage sludge to human salmonella isolates in Paper IV were stricter than the model set by Tenover *et al*. (1995). As a complement to PhenePlate analysis of VRE in Paper V, on the other hand, only one enzyme was used, but the criteria for indistinguishable strains were still stricter than suggested by Tenover *et al*. (1995).

PhenePlate analysis

PhenePlate analysis (PhenePlate™, PhPlate Microplate Techniques AB, Stockholm, Sweden) is a semi-automated biochemical fingerprinting method based on 24 different biochemical tests (PhPlate-FS) and suitable for enterococcal bacteria (Kuhn *et al.*, 1995). The bacterial suspensions to be tested are inoculated in a 96-well ready-made microtitre plate, which enables four samples to be tested per plate. An optical microplate reader is then connected to a computer and the unweighted-pair group method using average linkages (UPGMA) is used for cluster analysis with the PhenePlate software (Saeedi *et al.*, 2005).

Epidemiological investigations

Determination of MICs followed by comparison of antibiograms (Papers IV and V), as well as PFGE (Papers I, II, IV and V) and PhenePlate analysis (Paper V) were all used as tools for discriminating isolates from each other. For

epidemiological investigations, the PhenePlate method still needs to be complemented with PFGE or another genotyping method (Kuhn *et al.*, 1995). In Paper IV, salmonella isolates from sewage sludge (Paper I) were compared with salmonella isolates from salmonella-infected humans (paper IV). The comparison was performed by molecular typing based on PFGE, strengthened by antimicrobial resistance analysis. Using antibiograms to discriminate between strains works well as a complement to other typing methods, but it is not suitable for epidemiological investigations that exceed several years (Ruiz *et al.*, 2003). PFGE was also used to identify strains persisting in the sewage treatment plants (Papers I, II, IV and V).

PCR and 16S rRNA sequencing

The molecular genetic methods PCR and 16S rRNA sequencing were used as descriptive methods in typing enterococci (Paper V). The PCR method was the tool used to detect the genes *vanA* or *vanB* in vancomycin resistant enterococcal isolates and the 16S rRNA sequencing was used as a method for typing enterococcal species when the biochemical tests were unclear.

Main results

Occurrence of pathogens in untreated biowaste and raw sewage sludge

The investigations of the substrate in biogas plants and sewage sludge in WWTPs revealed a difference in the occurrence of pathogens in these untreated substrates (Paper I and II) (Table 4). There was more frequent isolation of bacterial pathogens (except *E. coli* O157) from raw sewage sludge from WWTPs than from untreated biowaste from biogas plants. The results from the pathogen analysis of samples from biogas plants and WWTPs are presented in Table 4.

Table 4. Pathogen content in substrate used at biogas plants and sewage sludge (Papers I and II). The treatment of sewage sludge consisted of either thermophilic or mesophilic treatment, windrow composting or sedimentation. Biowaste treatment consisted of pasteurisation and anaerobic digestion. Numbers of positive samples isolated are shown in brackets

	Substrate from biogas plants (n*=24)		Sewage sludge from WWTP (n=64)	
Pathogen	Untreated	Treated	Untreated	Treated
Salmonella spp.	17% (4)	0% (0)	67% (43)	55% (38)
L. monocytogenes	4% (1)	0% (0)	12% (8)	2% (1)
E. coli O157	8% (2)	0% (0)	2% (1)	0% (0)
C. coli/jejuni	25% (6)	0% (0)	30% (19)	5% (3)

*Number of samples

The hygiene quality in treated biowaste and sewage sludge

The analysis of treated sewage sludge (Paper I) and digested residue (Paper II) and the study of pasteurisation (Paper III) revealed differences in inactivation of pathogens and indicator bacteria between different treatments. There was an effective reduction of pathogens and indicator bacteria, except for spore-forming bacteria, after pasteurisation and digestion of the biowaste in the biogas plants (Table 4) (Paper II). Effective inactivation of pathogens and indicator bacteria was also observed in the laboratory study of pasteurisation (70°C, 60 min.) (Paper III). However, both spore-forming *Cl. perfringens* and heat resistant viruses (parvovirus) survived pasteurisation at 70°C for 60 min. (Paper III). In contrast to the digested residue in biogas plants, the survey of Swedish WWTPs demonstrated 55% salmonella-positive samples in treated sewage sludge (Paper I). The most commonly isolated *Salmonella* serotype was *S*. Hadar (Paper I). Thermophilic treatment was more effective in reducing pathogens and indicator bacteria than mesophilic anaerobic digestion (Paper I). *Salmonella* Stanley was the only pathogen isolated, on one occasion, after the thermophilic anaerobic digestion. This salmonella-positive sample was not taken directly after the digestion chamber but after the centrifugation of the thermophilically digested sewage sludge. The content of pathogens and indicator bacteria was not affected after sedimentation (Paper I).

Composted sewage sludge

The analysis of samples from windrow composts gave very varied results as regards indicator bacteria (Table 5) and the only isolate of *Campylobacter coli* from treated sewage sludge was found in windrow compost (Paper I).

Table 5. Number (CFU/g) of indicator bacteria in samples from windrow compost regarded as stabilised and ready for use (associated with Paper I)

	Entero-cocci	Coliforms	Thermo-tolerant coliforms	Presumptive *E. coli*	*Clostridium perfringens*
N*	7	8	8	8	7
Min	0	0	0	0	9 000
Max	640 000	1 520 000	180 000	180 000	3 000 000
Mean	108 928	190 535	25 500	24 500	516 857
SD	236 019	537 185	62 989	63 080	1 100 827

*Number of samples

Antimicrobial resistant bacteria in sewage sludge

Of the *Salmonella* spp. isolated from sewage sludge, 12% of the strains (12/101) were resistant to one or more antimicrobials and 7% were multiresistant (resistant to ≥ 3 antimicrobials) (Paper IV). Moreover, vancomycin resistant enterococci occurred in mesophilically digested sewage sludge every week during a 4-month long investigation (Paper V).

Molecular epidemiological relationships between strains from sewage sludge and other sources

PFGE patterns of six (6) pairs of *Salmonella* isolates from human cases of *Salmonella* infection and isolates from sewage sludge had indistinguishable PFGE patterns that were confirmed by antimicrobial resistance analysis (Paper IV).
In contrast, no connection was found between VRE isolated from sewage sludge and VRE isolates from Swedish broilers or from humans assessed by PhenePlate analysis. The results of the PhenePlate analysis were strengthened by PFGE and by differences in antibiograms (Paper V).

The epidemiological investigations (using PFGE, antibiograms and PhenePlate analysis) revealed that strains of *Enterococcus* spp. and *Salmonella* spp. persist in sewage sludge (Papers I, IV and V) and that the same *Salmonella* strain can be re-isolated for up to two years.

General discussion

Occurrence of pathogens in sewage sludge and biowaste

Salmonella

The results from the survey of WWTPs in Sweden (Paper I) revealed a high frequency of *Salmonella* spp. in raw and treated sewage sludge. Already in 1977, Danielsson reported a high frequency of *Salmonella* spp. in Swedish sewage sludge. The frequency of *Salmonella* spp. found in the sludge is at first sight remarkable, considering the reported low number of Swedish *Salmonella* cases in humans.
Salmonella is a notifiable disease according to the Communicable Disease Act in Sweden and less than 5000 human *Salmonella* infections are reported each year. Some 80% of the cases reported involved patients that were infected abroad (Anonymous, 2001). These figures are similar to findings regarding human *Salmonella* infections in Norway (Kapperud *et al.*, 1998). However, underreporting is a fact (Wheeler *et al.*, 1999) and the actual numbers of salmonella-infected carriers are possibly larger than assumed. Thus, the high frequency of *Salmonella* spp. in Swedish sewage sludge can in part be explained by a higher frequency of salmonella-infected humans served by the WWTP than reported, and/or by the infected people shedding the bacteria for a long period of time. However, as demonstrated in Papers I and IV, an additional cause of the high salmonella load in sewage sludge is most likely the ability of *Salmonella* spp. to persist and multiply in the WWTPs.

Sewage sludge is thought to reflect the health status of the human population served by a WWTP, shown for example by Jones *et al.*, (1990), who demonstrated a correlation between seasonal variations in human campylobacteriosis and campylobacter isolations in raw sewage sludge. The specific link shown in the present study between *Salmonella* strains from salmonella-infected humans and

Salmonella isolates from sewage sludge (Paper IV) demonstrates that *Salmonella* spp. in sludge originate from the human population served by the actual WWTP. This was shown by PFGE analysis and antibiogram typing of *Salmonella* strains from humans and sewage sludge, where PFGE patterns were indistinguishable for all three restriction enzymes used, and where the antibiograms were indistinguishable.

Our frequent findings of *Salmonella* spp. in Swedish sewage sludge (Paper I) were substantially higher than the findings from a similar Norwegian study reported by (Rosef, 1999). This is in contrast to the expected similarity suggested by similar reported numbers of human *Salmonella* cases. However, when the numbers of *Salmonella* findings are correlated to the number of persons served by the WWTPs studied, the Swedish results are comparable to the Norwegian results. This observation underlines the need to take into account the size of the WWTP when relating salmonella findings in sewage sludge to the health status of the population served. As Larsen (1995) points out, the number of persons served by a WWTP is directly related to the likelihood of *Salmonella* contamination.

Sewage sludge may act as a reservoir of *Salmonella* spp. and other pathogens, and insufficiently treated and recycled sewage sludge may enhance the load of *Salmonella* spp. in the society (Paper I). In Paper IV, it was demonstrated that salmonella contamination in sewage sludge was caused to a large extent by *Salmonella* strains that originated from abroad. Therefore recirculation of insufficiently (mesophilically) digested sewage sludge could enable 'imported' *Salmonella* strains to be transmitted to animals and humans and increase the domestic *Salmonella* infections in Swedish communities. There were 49 different *Salmonella* serotypes represented among the isolates from sewage sludge. However, the *Salmonella* serotypes found in sewage sludge (Papers I and IV) did not entirely correlate with the serotypes commonly reported among humans (Anonymous, 2001) although it is assumed that sewage sludge reflects the human health status. *Salmonella* Hadar was the most common serotype isolated from sewage sludge in this study (Paper I) but this serotype was not isolated in the studies conducted by Danielsson in the 1970s (Danielsson, 1977). The trend of *Salmonella* serotypes causing salmonella infection in humans has not changed during the last 20 years (Birgitta de Jong, pers. comm., 2006). It is still *S.* Enteritidis among travellers and *S.* Typhimurium that cause the most commonly reported human *Salmonella* infections in Sweden (Paper IV). *Salmonella* Hadar was also the most common serotype in sewage reported in a Spanish study and there too, the findings did not reflect the situation in reported human salmonella cases among Spanish citizens (Espigares *et al.*, 2006). This supports our theory (Paper IV) that some serotypes have a better ability to survive the treatment in WWTPs. However, it is important to maintain detailed monitoring of human salmonella cases that includes both serotype and origin, in order to identify a change in domestic salmonella cases in particular that could indicate a spread of *Salmonella* spp. from sources such as sewage sludge.

The results from the surveys of biogas plants and WWTPs in Sweden (Papers I and II) revealed differences in the content of pathogens in raw sewage sludge and untreated biowaste. This difference was mainly caused by the frequent isolation of

Salmonella spp. in sewage sludge. There is a low prevalence of salmonella infection among Swedish livestock, a fact reflected in the biowaste from the biogas plants, which includes high proportions of manure. The different hygiene quality in the different types of wastes implies that it is wise to keep the substrates separate, so as not to contaminate the biowaste with the more infected sewage sludge. The cleaner the raw materials are, the better the hygiene quality one can expect from the treated product. However, there may be other benefits with mixing materials, for example to improve the C/N ratio in a compost process.

Campylobacter

Campylobacter, together with salmonella, is the most common bacterial cause of gastroenteritis in humans. However compared to salmonella, campylobacter were rarely isolated from sewage sludge (Paper I) and biowaste (Paper II) in our studies (Table 4). *Campylobacter* need microaerophilic growth conditions, rapidly transform to a viable but non-culturable state and may be hard to detect in environmental samples (Talibart, 2000; Jones, 2001; Abulreesh *et al.*, 2005; Gallay *et al.*, 2006). In Paper III, biowaste samples were seeded with *C. jejuni* in a pilot case of the laboratory pasteurisation study but the bacteria could not be re-cultivated from the untreated substrate (data not shown). Lack of appropriate selective culture media for Campylobacter spp. may result in that other bacteria outgrow *Campylobacter* spp. in routine laboratory analysis procedures (Abulreesh *et al.*, 2005).These analytical difficulties may be the reason for the low detection rate of the bacterium in our studies. This is in agreement with the inability to isolate campylobacter from sewage sludge reported by Jones *et al*. (1990). However in 1998, the same authors found 8% *C. jejuni* positive samples taken from sludge spread on land (Jones, 2001). If there is a lack in detection of *Campylobacter* spp. in sewage sludge, the sludge could contain this bacterial species in larger quantities than our analysis encountered. This would imply that *Campylobacter* spp. may be a larger risk factor in sewage sludge than believed. Further research is needed to evaluate and develop methods for isolating *Campylobacter* spp. from environmental samples.

Antimicrobial resistance

Antimicrobial resistance in *Salmonella* spp. occurred in 12% of the sewage sludge strains tested (Paper IV). There are few other studies of sewage sludge to compare with, but a Spanish and a Finnish study of sewage revealed higher frequencies of resistance in isolated *Salmonella* spp., almost 70% and 44%, respectively (Koivunen *et al.*, 2003; Espigares *et al.*, 2006). These figures indicate a lower frequency of resistant *Salmonella* spp. in Sweden compared to other countries. This could be a result of the restrictive antibiotic policy in Sweden.

In addition to antimicrobial resistant *Salmonella* spp., there are VRE in sewage sludge that are also able to survive mesophilic anaerobic digestion (Papers IV and V). The epidemiological studies comparing human, sewage and chicken strains of VRE did not reveal any connection between these strains (Paper V). VRE found in sewage sludge were mainly *E. faecium* harbouring the *vanB* gene, in agreement with the majority of clinical human cases in Sweden (SWEDRES 2005, 2006).

The commonly isolated strain in Swedish broilers is *E. faecium* carrying the *vanA* gene (SVARM, 2005). This observation indicates that the VRE in broilers in Sweden, which most likely have a clonal origin (SVARM, 2005), are not the source of human VRE infection.

PhenePlate analysis seemed to be a good tool as earlier described by Kuhn *et al.* (1995) for a first rapid screening of a large number of enterococci. This was confirmed by PFGE analysis to verify the relationships (Paper V). In contrast with Saeedi *et al*. (2005), the results from our analysis of *E. faecium* with the PhenePlate technique in Paper V were well correlated with the PFGE analysis.

The demonstrated ability of VRE and *Salmonella* spp. to persist and possibly multiply in the sewage sludge in the WWTP (Papers IV and V) indicates that sewage sludge may act as a reservoir of antimicrobial resistant bacteria. The frequent occurrence of VRE and multiresistant *Salmonella* implies a need for a more efficient hygiene treatment of sewage sludge, in order to avoid the spread of antimicrobial resistance through the use of sewage sludge. A more effective treatment such as thermophilic anaerobic digestion or pasteurisation that inactivates enterococci and *Salmonella* spp. would also inactivate the bacteria harbouring resistance genes and would most likely make it impossible for genetic transfer of antimicrobial resistant genes. More research is needed regarding whether resistance genes retain the ability to be transfered into bacteria after their original bacteria have lysed.

Sewage released to recipient waters and sewage sludge spread on arable land may enhance the spread of resistant strains in our environment. Antimicrobial resistant bacteria may interact with, and transfer resistance genes to, other bacteria in water and soil (Agerso & Sandvang, 2005) and the load of antimicrobial resistant bacteria in our society may increase. In addition, the risk of genetic elements persisting and being transferred to vegetative bacteria must not be ruled out and the possible transmission of resistance genes should be further studied.

Differences in treatments regarding sanitation

Pasteurisation

From a hygiene point of view, pasteurisation in combination with anaerobic digestion of biowaste in biogas plants resulted, as expected, in a better residue than treated sewage sludge from WWTPs using conventional treatments (Papers I and II). Pasteurisation is not used at WWTPs in Sweden today, in part because it is more expensive and technically demanding. If it were used, the hygiene quality of the sewage sludge would increase remarkably. However, results from the laboratory study of pasteurisation revealed that heat resistant viruses such as parvovirus and spore-forming bacteria such as *Cl. perfringens* were able to survive at 70°C for 1 h. The spore-formers in sewage sludge are believed to cause no problems, but when manure, which more often contains *e.g. Cl. chauvoei*, is treated in biogas plants, the result can be spread of diseases to areas that were previously free from the infection. In addition, there is still a possibility that other pathogens not analysed for (such as heat resistant viruses) may survive

pasteurisation and possibly cause problems. In Sweden, there is a recommendation not to spread digested residue on pasture in order to avoid infection of grazing animals.

A change in the present treatment requirements (EC 1774/2002) for animal by-products is to be implemented from January 2007, which will enable other treatments that are equally effective as compared to pasteurisation to be used (EC Directive 208/2006). Individual EU member states will be required to perform a validation and evaluation of new techniques before they can be approved.

Anaerobic digestion

In agreement with previous studies (Smith *et al.*, 2005), thermophilic anaerobic digestion can be recommended as a better treatment of sewage sludge than anaerobic digestion in mesophilic temperatures in terms of pathogen inactivation (Paper I). A general change of process temperature from mesophilic to thermophilic anaerobic digestion would remarkably decrease the content of pathogenic bacteria in sewage sludge in Swedish WWTPs. However, one has to bear in mind that thermophilic anaerobic digestion will not result in disinfected sewage sludge. Continuous digestion compared to batch-wise anaerobic digestion increases the risk of insufficient retention time in the digestor, which could result in insufficient inactivation of pathogens. Some pathogens may survive, for example *Salmonella* Stanley was isolated after centrifugation in the WWTP that used thermophilic anaerobic digestion (Paper I). This is not a unique finding, since insufficient inactivation of *Salmonella* spp. in thermophilic anaerobic digestion has been reported by *e.g.* Zabranska *et al*. (2003). Parvovirus also has the ability to resist thermophilic anaerobic digestion (Lund *et al.*, 1996). In addition, there may be other heat resistant human, animal or plant pathogens not studied here that may survive thermophilic anaerobic digestion. However, the thermophilic anaerobic digestion of sewage sludge would most likely inactivate human and animal pathogens to such a degree that the risk of infectious doses associated with controlled use of the treated product in the environment could be avoided.

Storage and windrow composting

Windrow composting may in some circumstances (inadequate maintenance such as too few turnings) resemble storage rather than composting. The product quality should be assured based upon a system of quality management (Godfree & Farrell, 2005). In windrow composts, the process control should preferably be based on monitoring time and temperature. Time is easily measured as long as the compost is processed batch-wise. Temperature may be more difficult to monitor in an open-air windrow because of the large variation of temperature in different areas in the large heap. The large variations in the results from analysis of windrow compost samples (Table 5) could have been caused by uneven mixing of the material and temperature differences in the large heap resulting in areas with less sanitation.

On the other hand, the variable results (Table 5) could be due to inappropriate sampling procedures. Representative samples of large windrows and heaps are difficult to obtain when only a 25 g sample is used *e.g.* for *Salmonella* analysis.

The sampling method used for the windrow compost does not give representative samples from a whole batch of composted material and is more like a spot test. This brings a bias in the analysis and results of the hygiene quality.

Product control of sewage sludge in windrows and heaps needs an improved method of obtaining representative samples from a large batch. Until the sampling procedure is revised and refined so that representative samples can be taken, the main issue should be to treat the sewage sludge and biowaste so that product control is less important. Storage and windrow composting must be defined as treatment methods where duration, operating conditions and maintenance must be strictly defined in order to get uniformly acceptable quality of the sewage sludge which would fulfil hygiene requirements set by different authorities. If the treatment used is well-documented and reliable, the process control (such as assessing time, temperature or other process parameters) could more or less replace the product control. However, an efficient product control could identify a possible recontamination of the treated product.

In full-scale biogas plants, the issue of temperature monitoring must be addressed, especially when additional heat in the form of *e.g.* heated steam is added at the top of the chamber. Variations in the temperature in the large volume of substrate may occur.

Recontamination

The handling of treated biowaste and sewage sludge is critical, so as to avoid re-contamination of the sanitised material. The survey of the biogas plants (Paper II) highlighted the importance of keeping treated material separate from untreated substrate. Treated and untreated substrate should also be handled with separate equipment and vehicles to avoid cross-contamination. In Paper II, the amount of indicator bacteria increased in the pasteurised substrate after anaerobic digestion and subsequent storage, possibly because of recontamination or due to an inefficient inactivation of bacteria. Furthermore, the ability of bacteria to transfer into a viable but non-culturable state may be a cause of the re-isolation of bacteria in digested residue in the storage wells (Paper II).

Different treatments render the sanitised material more or less prone to recontamination. Chemical treatment with urea has been demonstrated to eliminate the risk of regrowth as long as the ammonia remains in the substrate (Vinnerås *et al.*, 2003). Sidhu *et al*. (2001) assumed the indigenous microflora to be the main cause of the suppression of *Salmonella* spp. re-growth in composted sewage sludge. They recommend that composted material should be stored for as short a time as possible in order to avoid re-growth in the material, because of a decline in the indigenous microflora during storage (Sidhu *et al.*, 2001). It is important that workers handling biowaste and sewage sludge are aware of the fact that they are working with contagious material, and of the risks of recontamination of sanitised material.

Significance and impact of the study

Sewage sludge and biowaste studied in this thesis were contaminated with different kinds of pathogens contagious both for animals and humans. Furthermore, sewage sludge was found to contain antimicrobial resistant bacteria, which are an additional risk for human and animal health.

The substrate used at full-scale biogas plants in Sweden is less contaminated with bacterial pathogens and the quality of digested residue from biogas plants is better from a hygiene point of view than that of sewage sludge from WWTPs. The hygiene quality of sewage sludge could be improved if mesophilic anaerobic digestion were replaced to a greater extent with thermophilic anaerobic digestion, because thermophilic (50-55°C) treatment is much more effective in reducing pathogens. Furthermore, a pre-pasteurisation step at the WWTPs would decrease the pathogen load, so that the risk for infection with arable use of sewage sludge could be minimised.

Salmonella spp. and VRE from humans that are passed through the sewage treatment system may multiply in sewage sludge and persist for long periods of time, up to two years in case of *Salmonella* spp. Via the sewage sludge, pathogens resistant to antimicrobials may be able to spread in the environment and enhance the environmental load of antimicrobial resistant pathogens. This may increase the risk of the environment acting as a reservoir of antimicrobial resistance.

However, a hygienically acceptable product is not impossible to achieve, but it is important to be aware of the infection risk and to treat the biowaste and sewage sludge in such a way that the pathogens are inactivated and the risk of infection is eliminated or minimised.

Further research

The results from the work in this thesis demonstrate that biowaste and sewage sludge in particular contain pathogens that if not efficiently eliminated before use could cause health risks for animals and humans. However, the relative proportions of the risks were not analysed. It would be of great interest to carry out a risk analysis for infection of animals and humans associated with the use of biowaste and sewage sludge as fertiliser. This would involve investigation of the survival of pathogens from biowaste and sewage sludge when they reach the soil via contaminated fertiliser, and how environmental factors impact on their inactivation. Moreover, it would be interesting to analyse bacterial multiplication in the environment, especially in sewage sludge, water and soil, and to scrutinise the different conditions that affect multiplication and the degree to which they affect multiplication of pathogens regarding *e.g*. storage of sewage sludge.

Future research regarding antimicrobial resistant bacteria might include studies on their ability to transfer genetic information to other bacteria in sewage sludge. It would also be interesting to study whether transferable genetic elements from inactivated antimicrobial resistant bacteria are transferred to other bacteria in sewage sludge or further to bacteria in soil and water, and the frequency with which this might occur.

Sampling techniques need to be further improved to allow trustworthy results to be obtained from product control of large quantities of material. Process and product control systems also need to be developed and evaluated in order to meet the need for new upcoming techniques for sewage sludge and biowaste treatment.

References

Abulreesh, H. H., Paget, T. A. & Goulder, R. (2005) Recovery of thermophilic campylobacters from pond water and sediment and the problem of interference by background bacteria in enrichment culture. *Water Research,* 39, 2877-82.

Ad-Nett (2000) *Anaerobic Digestion; Making energy and solving modern waste problems.* Ad-Nett Report 2000, (Ed. Örtenblad, H.), pp. 195. http://www.adnett.org

Agerso, Y. & Sandvang, D. (2005) Class 1 integrons and tetracycline resistance genes in alcaligenes, arthrobacter, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Applied and Environmental Microbiology,* 71, 7941-7.

Andersson, D. I. (2003) Persistence of antibiotic resistant bacteria. *Current Opinion in Microbiology,* 6, 452-6.

Anonymous (2001) Communicable diseases in Sweden 2001, Annual report of Department of Epidemiology, Swedish Institute for Infectious Disease Control, Solna. http://www.smittskyddsinstitutet.se/upload/publikationer /Report2001.pdf 9-Aug-2006.

Anonymous (2005) Epi-Aktuellt 38:2005, Swedish Institute for Infectious Disease Control, Solna. www.smittskyddsinstitutet.se

Anonymous (2006) Epidemiologisk Årsrapport 2005. SMI-tryck 157-2006 Swedish Institute for Infectious Disease Control, Solna, ISSN 3473. http://www.smittskyddsinstitutet.se/upload/Publikationer/SMI-epidemiologisk-arsrapport-2005.pdf 9-Aug-2006

Avery, L. M., Killham, K. & Jones, D. L. (2005) Survival of *E. coli* O157:H7 in organic wastes destined for land application. *Journal of Applied Microbiology,* 98, 814-22.

Bagge, E., Sahlström, L. & Albihn, A. (2005) The effect of hygienic treatment on the microbial flora of biowaste at biogas plants. *Water Research,* 39, 4879-86.

Bendixen, H. J. & Ammendrup. S. (1992) Safeguards against pathogens in biogas plants. Veterinary research, monitoring and consulting on establishment and operation of joint biogas plants. Ministry of Agriculture, Danish Veterinary Service.

Berggren, I., Albihn, A. & Johansson, M. (2004) The effect of temperature on the survival of pathogenic bacteria and *Ascaris suum* in stored sewage sludge *11th Int. Conf. of the FAO ESCORENA Network on the Recycling of Agricultural, Municipal and Industrial Residues in Agriculture.* Murcia, Spain.

Berggren, I., Albihn, A. & Johansson, M. (2005) Långtidslagring av avloppsslam-effekt på hygienisk kvalitet. The effect of long term storage of sewage sludge- sanitary quality. *VA-forsk rapport 2005-04,* Svenskt Vatten, Stockholm.

Berndtson, E. (1996) *Campylobacter in broiler chickens.* The mode of spread in chicken flocks with special reference to food hygiene. Faculty of Veterinary Medicine, the department of food hygiene. Uppsala, Swedish University of Agricultural Sciences, Ph.D. thesis. ISBN-91-576-5104-3.

Bille, J. (1996) An overview of *Listeria monocytogenes*. In *Food Associated Pathogens.* Proceedings of the symposium Food Associated Pathogens, May 6-8, 1996, Uppsala, Sweden, Department of Food Hygiene, SLU, pp. 82-85, ISBN 91-576-5132-9.

Bitton, G. (1999) *Wastewater microbiology*. 2nd edition. Wiley-Liss, New York.

Carducci, A., Arrighi, S. & Ruschi, A. (1995) Detection of coliphages and enteroviruses in sewage and aerosol from an activated sludge wastewater treatment plant. *Letters in Applied Microbiology,* 21, 207-9.

Carducci, A., Gemelli, C., Cantiani, L., Casini, B. & Rovini, E. (1999) Assessment of microbial parameters as indicators of viral contamination of aerosol from urban sewage treatment plants. *Letters in Applied Microbiology,* 28, 207-10.

Carlander, A. (2006) *Assessment of microbial health hazards associated with wastewater application to willow coppice, coniferous forest and wetland systems,* Uppsala, Dept. of Crop Production Ecology Swedish University of Agricultural Sciences, Ph.D. thesis. ISSN 1652-6880, ISBN 91-576-7078-1.

Carlander, A., Aronsson, P., Allestam, G., Stenström, T. A. & Perttu, K. (2000) Transport and retention of bacteriophages in two types of willow-cropped lysimeters. *Journal of Environmental Science and Health.* A35, 1477-92.

Carrington, E. G. (2001) Evaluation of sludge treatments for pathogen reduction-Final report. European commission. Report No 5026/1.

Cars, O., Molstad, S. & Melander, A. (2001) Variation in antibiotic use in the European Union. *Lancet,* 357, 1851-3.

Carter, G. R. & Chengappa, M. M. (1991) *Essentials of veterinary bacteriology and mycology,* Philadelphia, Lea & Febiger.

Clements, R. P. L. (1983) Sludge hygienization by means of pasteurisation prior to digestion. In *Disinfection of sewage sludge: Technical, economic and microbiological aspect, proceedings of a workshop, pp. 37-52. (*Eds. A. M. Bruce, A. H. Havelaar & P. L. L´Hermite), *Zurich, May 11-13, 1982.* Dordrecht, Holland.

Cookson, B. (2005) Clinical significance of emergence of bacterial antimicrobial resistance in the hospital environment. *Journal of Applied Microbiology,* 99, 989-96.

Cotruvo, J. A., Rees, A. D. G., Bartram, J., Carr, R., Cliver, D. O., Craun, G. F., Fayer, R. & Gannon, V. P. J. (Eds.) (2004) *Waterborne zoonoses: identification, causes, and control,* London, IWA Publishing.

Courvalin, P. (2005) Genetics of glycopeptide resistance in gram-positive pathogens. *International Journal of Medical Microbiology,* 294, 479-86.

Danielsson, M.-L. (1977) *Salmonella in sewage and sludge; serological profiles of isolates, their removal and/or survival in relation to potential health hazards to man and animals*. Acta Veterinarica Scandinavica, Suppl 65, Ph.D. thesis. ISSN 91-7088-2, ISBN 0065-1699.

Dasgupta, A. P. & Hull , R. R. (1989) Late blowing of Swiss cheese: Incidence of *Clostridium tyrobutyricum* in manufacturing milk. *Australian Journal of Dairy Technology,* 44, 82-7.

D'Aoust, J-Y, Maurer, J. & Bailey, J. S. (2001) *Salmonella* species. In *Food microbiology: fundamentals and frontiers.* (Eds. M. P. Doyle, L. R. Beuchat, & T. J. Montville)*,* Washington, DC, ASM Press, pp. 141-78.

de Jong, B. (2006), epidemiologist, Smittskyddsenheten, Norrbacka, 171 76, Stockholm, personal communication, July 2006.

De Luca, G., Zanetti, F., Fateh-Moghadm, P. & Stampi, S. (1998) Occurence of *Listeria monocytogenes* in sewage sludge. *Zentralblatt für Hygiene und Umweltmedizine,* 201, 269-77.

Dorn, C. R. (1993) Review of foodborne outbreak of *Escherichia coli* O157:H7 infection in the western United States. *Journal of the American Veterinary Medical Association,* 203, 1583-7.

Dudley, D. J., Guentzel, M. N., Ibarra, M. J., Moore, B. E. & Sagik, B. P. (1980) Enumeration of potentially pathogenic bacteria from sewage sludges. *Applied and Enviromental Microbiology,* 39, 118-26.

Dumontet, S., Dinel, H. & Baloda, S.B. (1999) Pathogen reduction in sewage sludge by composting and other biological treatments: A review. *Biological Agriculture & Horticulture,* 16, 409-30.

Dumontet, S., Scopa, A., Kerje, S. & Krovacek, K. (2001) The importance of pathogenic organisms in sewage and sewage sludge. *Journal of the Air & Waste Management Association,* 51, 848-60.

EC 1774/2002, *Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption*, http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32002R1774:EN:HTML, 10-Aug-2006.

Eller, G., 1995. *Liquid composting of raw wastewater, mixed with biodegradable waste. Persistence of selected pathogens and indicator organisms.* The Institute for Sanitary Engineering. Brunswick: Technical University of Brunswick.

Emmoth, E., Dergel, I., Mcneilly, F., Allan, G. M., Albihn, A. & Klingeborn, B. (2004) Heat inactivation of Porcine circovirus type 2. In *FAO European Cooperative Research. Ramiran: Sustainable Organic Waste Management for Environmental Protection and Food Safety* (Eds. Bernal, M. P., Clemente, R. M. R. & Paredes, C.) Murcia, Spain, Tipografia San Francisco, S.A.

Endo, T. & Morishima, Y. (2004) Major helminth zoonoses in water. In *Waterborne zoonoses: identification, causes, and control.* (Eds. J. A. Cotruvo, A. D. G. Rees, J. Bartram, R. Carr, D. O. Cliver, G. F. Craun, R. Fayer & V. P. J. Gannon) London, IWA Publishing, pp. 291-304.

Espensen, B. (1996) Praktiske forsög med smitstofreducerende bahandling af husholdningsaffald. *Dansk Veterinaer Tidsskrift,* 79, 615-22.

Espigares, E., Bueno, A., Espigares, M. & Galvez, R. (2006) Isolation of *Salmonella* serotypes in wastewater and effluent: Effect of treatment and potential risk. *International Journal of Hygiene and Environmental Health,* 209, 103-7.

EUCAST Procedure for Harmonising and Defining Breakpoints http://www.srga.org/Eucastwt/bpsetting.htm, 8-Aug-2006.

Fayer, R. (2004) Waterborne zoonotic protozoa. In *Waterborne zoonoses: identification, causes, and control.* (Eds. J. A. Cotruvo, A. D. G. Rees, J. Bartram, R. Carr, D. O. Cliver, G. F. Craun, R. Fayer & V. P. J. Gannon) London, IWA Publishing, pp. 255-82.

Feachem, R. G. (1983) *Sanitation and disease: health aspects of excreta and wastewater management,* Chichester, Wiley.

Fredriksson-Ahomaa, M., Stolle, A., Siitonen, A. & Korkeala, H. (2006) Sporadic human *Yersinia enterocolitica* infections caused by bioserotype 4/O: 3 originate mainly from pigs. *Journal of Medical Microbiology,* 55, 747-9.

Gallay, A., De Valk, H., Cournot, M., Ladeuil, B., Hemery, C., Castor, C., Bon, F., Megraud, F., Le Cann, P. & Desenclos, J. C. (2006) A large multi-pathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. *Clinical Microbiology and Infection,* 12, 561-70.

Gaspard, P. G., Wiart, J. & Schwartzbrod, J. (1995) Urban sludge reuse in agriculture: Waste treatment and parasitological risk. *Bioresource Technology,* 52, 37-40.

Gerba, C. H., Rose, J. B. & Haas, C. N. (1996) Sensitive populations: who is at the greatest risk? *International Journal of Food Microbiology,* 30, 113-123.

Gibbs, R. A., Hu, C. J., Ho, G. E. & Unkovich, I. (1997) Regrowth of faecal coliforms and salmonellae in stored biosolids and soil amended with biosolids. *Water Science and Technology,* 35, 269-75.

Godfree, A. (2003) Health constraints on the agricultural recycling of wastewater sludges. In *The Handbook of Water and Wastewater Microbiology.* (Eds. D. Mara & N. Horan) Amsterdam, Academic Press, pp. 281-98.

Godfree, A. & Farrell, J. (2005) Processes for managing pathogens. *Journal of Environmental Quality,* 34, 105-13.

Grange, J. M. & Yates, M. D. (1994) Zoonotic aspects of *Mycobacterium bovis* infection. *Veterinary Microbiology,* 40, 137-51.

Guardabassi, L., Bronnum, P. T., Dano, R., Forslund, A. & Dalsgaard, A. (2002) Dissemination of vancomycin-resistant enterococci harboring *vanA* through disposal of waste derived from industrial production of vancomycin. *Microbial Drug Resistance,* 8, 401-6.

Harwood, V. J., Levine, A. D., Scott, T. M., Chivukula, V., Lukasik, J., Farrah, S. R. & Rose, J. B. (2005) Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. *Applied and Environmental Microbiology,* 71, 3163-70.

Heritage, J. (2003) Viruses. In *The Handbook of Water and Wastewater Microbiology.* (Eds. D. Mara & N. Horan)*,* Amsterdam, Academic Press, pp. 37-55.

Hirsh, D. C. & Zee, Y. C. (1999) *Veterinary Microbiology*, Blackwell Science, Inc.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (1994) *Bergeys Manual of Determinative Bacteriology*, Williams & Wilkins, Baltimore.

House, J. A. & House, C. A. (1999) Vesicular Diseases. In *Diseases of Swine*, 8th edition. (Eds. B. E. Straw, S. D'Allaire, W. L. Mengeling &. D. J. Taylor) Iowa State University Press pp. 327-340.

Höglund, C. (2001) *Evaluation of microbial health risks associated with the reuse of source-separated human urine,* Royal Institute of Technology, Department of Biotechnology, Stockholm, Ph.D. thesis. ISBN 91-7283-039-5.

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P. & Jiang, X. (2004) Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology,* 70, 2497-502.

Iversen, A., Kuhn, I., Franklin, A. & Möllby, R. (2002) High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Applied and Environmental Microbiology,* 68, 2838-42.

Jack, E. J. & Hepper, P. T. (1969) An outbreak of Salmonella typhimurium infection in cattle associated with the spreading of slurry. *The Veterinary Record,* 84, 196-9.

Johansson, M., Emmoth, E., Salomonsson, A. C. & Albihn, A. (2005) Potential risks when spreading anaerobic digestion residues on grass silage crops - survival of bacteria, moulds and viruses. *Grass and Forage Science,* 60, 175-185.

Johansson, P. O., Espeby, B., Nilsson, B. & Allestam, G., (1998) Artificial groundwater recharge in Stockholm - II Column test design and tracer tests. In: *Artificial Recharge of Groundwater*, (Eds. J. H. Peters *et al*.) Balkema AA, Rotterdam, pp. 383-5.

Jones, K. (2001) Campylobacters in water, sewage and the environment. *Journal of Applied Microbiology,* 90, 68S-79S.

Jones, K., Betaieb, M. & Telford, D. R. (1990) Seasonal variation of thermophilic campylobacters in sewage sludge. *Journal of Applied Bacteriology,* 69, 185-9.

Jones, P. W. (1980) Health hazards associated with the handling of animal wastes. *The Veterinary Record,* 5, 4-7.

Junttila, J. R., Niemela, S. I. & Hirn, J. (1988) Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. *Journal of Applied Bacteriology,* 65, 321-7.

Kapperud, G., Lassen, J. & Hasseltvedt, V. (1998) *Salmonella* infections in Norway: descriptive epidemiology and a case-control study. *Epidemiology and Infection,* 121, 569-77.

Kearney, T. E., Larkin, M. J., Frost, J. P. & Levett, P. N. (1993) Survival of pathogenic bacteria during mesophilic anaerobic digestion of animal waste. *Journal of Applied Bacteriology,* 75, 215-9.

Keller, U. (1983) Experiences and development of sludge paseurization in Altenrhein. In *Disinfection of sewage sludge: Technical, economic and microbiological aspect: proceedings of a workshop, pp. 53-67 (*Eds. A.

M. Bruce, A. H. Havelaar & P. L. L´Hermite)*, Zurich, May 11-13, 1982,* Dordrecht, Holland.

Khuder, S. A., Arthur, T., Bisesi, M. S. & Schaub, E. A. (1998) Prevalence of infectious diseases and associated symptoms in wastewater treatment workers. *American Journal of Industrial Medicine,* 33, 571-7.

Klare, I., Konstabel, C., Badstubner, D., Werner, G. & Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium. International Journal of Food Microbiology,* 88, 269-90.

Koivunen, J. & Heinonen-Tanski, H. (2005) Inactivation of enteric microorganisms with chemical disinfectants, UV radiation and combined chemical/ UV treatments. *Water Research,* 39, 1519-26.

Koivunen, J., Siitonen, A. & Heinonen-Tanski, H. (2003) Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units. *Water Research,* 37, 690-8.

Koopmans, M. & Duizer, E. (2004) Foodborne viruses: an emerging problem. *International Journal of Food Microbiology,* 90, 23-41.

Kudva, I. T., Blanch, K. & Hovde, C.J. (1998) Anaysis of *Escherichia coli* O157:H7 survival in ovine or bovine manure and manure slurry. *Applied and Environmental Microbiology,* 64, 3166-74.

Kühn, I., Burman, L. G., Haeggman, S., Tullus, K. & Murray, B. E. (1995) Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. *Jornal of Clinical Microbiology,* 33, 2812-7.

Larsen, H. E. (1995) Rev: Bakteriologiske risici ved anvendelse af husdyrgödning og affald. *Dansk Veterinaer Tidskrift,* 78, 763-6.

Larsen, H. E. & Munch, B. (1986) pathogenic bacteria in extra animal environments rev. *Ugeskrift for Jordbrug. Selected research reviews*, 57-65.

Larsen, H. E., Munch, B. & Schlundt, J. (1994) Use of indicators for monitoring the reduction of pathogens in animal waste treated in biogas plants. *Zentralblatt für Hygiene und Umweltmedizine,* 195, 544-555.

Leclerc, H., Edberg, S., Pierzo, V. & Delattre, J. M. (2000) Bacteriophages as indicators of enteric viruses and public health risk in groundwaters. *Journal of Applied Microbiology,* 88, 5-21.

Lilleengen, K. (1948) Typing of Salmonella typhimurium by means of a bacteriophage. The bacteriological and hygienical department, The Royal Veterinary College, Stockholm, Sweden.

Lund, B., Jensen, V. F., Have, P. & Ahring, B. (1996) Inactivation of virus during anaerobic digestion of manure in laboratory scale biogas reactors. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology,* 69, 25-31.

Mach, P. A. & Grimes, D. J. (1982) R-plasmid transfer in a wastewater treatment plant. *Applied and Environmental Microbiology,* 44, 1395-403.

Marcinek, H., Wirth, R., Muscholl-Silberhorn, A. & Gauer, M. (1998) *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. *Applied and Environmental Microbiology,* 64, 626-32.

Meng, X. J., Purcell, R. H., Halbur, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. (1997) A novel virus in swine is closely related to the human hepatitis E virus. *The Proceedings of the National Academy of Sciences USA,* 94, 9860-5.

Mitscherlich, E. A. & Marth., E. H. (1984) *Microbial Survival in the environment*, Springer-Verlag.

Moe, C. L. (2004) What are the criteria for determining whether a disease is zoonotic and water related? In *Waterborne zoonoses: Identification, causes, and control.* (Eds. J. A. Cotruvo, A. D. G. Rees, J. Bartram, R. Carr, D.O. Cliver, G.F. Craun, R. Fayer & V.P.J. Gannon) London, IWA Publishing, pp. 27-45.

Molbak, K. & Scheutz, F. (2004) Verocytotoxin-producing *Escherichia coli* and other diarrhoeagenic *E. coli*. In *Waterborne zoonoses: Identification, causes, and control.* (Eds. J. A. Cotruvo, A. D. G. Rees, J. Bartram, R. Carr, D. O. Cliver, G. F. Craun, R. Fayer & V. P. J. Gannon) London, IWA Publishing, pp. 213-27.

Nachamkin, I. (2001) *Campylobacter jejuni*. In *Food microbiology: fundamentals and frontiers.* (Eds. M. P. Doyle, L. R. Beuchat, & T. J. Montville), Washington, DC, ASM Press, pp. 179-92.

Natvig, E. E., Ingham, S. C., Ingham, B. H., Cooperband, L. R. & Roper, T. R. (2002) *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. *Applied and Environmental Microbiology,* 68, 2737-44.

Ogden, I. D., Hepburn, N. F., Macrae, M., Strachan, N. J., Fenlon, D. R., Rusbridge, S. M. & Pennington, T. H. (2002) Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. *Letters in Applied Microbiology,* 34, 100-4.

Office International des Epizooties, World Organisation of Animal Health. http://www.oie.int/eng/maladies/en_classification2005.htm, 8-Aug-2006.

Oliver, J. D. (2005) The viable but nonculturable state in bacteria. *Journal of Microbiology,* 43 Spec No, 93-100.

Olsen, J. E. & Larsen, H. E. (1987) Bacterial decimation times in anaerobic digestions of animal slurries. *Biological Wastes,* 21, 153-168.

Palmgren, H. (2002) *Importance of wild birds in the spread of Salmonella,* Umeå, Univ, Ph.D. thesis. ISSN 0346-6612 ISBN 91-7305-255-8 .

Pickup, R. W., Rhodes, G., Bull, T. J., Arnott, S., Sidi-Boumedine, K., Hurley, M. & Hermon-Taylor, J. (2006) *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: diverse opportunities for environmental cycling and human exposure. *Applied and Environmental Microbiology,* 72, 4067-77.

Reilly, W. J., Forbes, G. I., Paterson, G. M. & Sharp, J. C. M. (1981) Human and animal salmonellosis in Scotland associated with environmental contamination, 1973-1979. *The Veterinary Record,* 108, 553-5.

Robins-Browne, R. M. (2001) *Yersinia enterocolitica*. In *Food microbiology : fundamentals and frontiers.* (Eds. M. P. Doyle, L. R. Beuchat, & T. J. Montville), Washington, DC, ASM Press, pp. 383-409.

Rosef, O. (1999) *Salmonella* i avlopsslam (*Salmonella* in sludge). *Norsk veterinaertidskrift,* 111, 795-9.

Ruiz, M., Rodriguez, J. C., Sirvent, E., Escribano, I., Cebrian, L. & Royo, G. (2003) Usefulness of different techniques in the study of the epidemiology of salmonellosis. *Apmis,* 111, 848-56.

Saeedi, B., Tarnberg, M., Gill, H., Hallgren, A., Jonasson, J., Nilsson, L. E., Isaksson, B., Kühn, I. & Hanberger, H. (2005) Phene Plate (PhP) biochemical fingerprinting. A screening method for epidemiological typing of enterococcal isolates. *Apmis,* 113, 603-12.

Sahlström, L. (2003) A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Bioresource Technology,* 87, 161-6.

Sahlström, L., Aspan, A., Bagge, E., Tham, M. L. & Albihn, A. (2004) Bacterial pathogen incidences in sludge from Swedish sewage treatment plants. *Water Research,* 38, 1989-94.

Sahlström, L., De Jong, B. & Aspan, A. (2006) *Salmonella* isolated in sewage sludge traced back to human cases of salmonellosis. *Letters in Applied Microbiology* 43, 46-52.

SCB (2004) Utsläpp till vatten och slamproduktion 2002. English title: Discharges to water and sludge production 2002. Sveriges Officiella Statistik Statistiska Meddelanden, MI-Miljövård, MI 22 SM 0401, Statistiska cebtralbyrån & Naturvårdsverket, ISSN 1403-8978.

Schroeder, E. & Wuertz, S. (2003) Bacteria. In *The Handbook of Water and Wastewater Microbiology*. (Eds. D. Mara & N. Horan) Amsterdam, Academic Press, pp. 57-68.

Schwartz, D. C., Saffran, W., Welsh, J., Haas, R., Goldenberg, M. & Cantor, C. R. (1983) New techniques for purifying large DNAs and studying their properties and packaging. *Cold Spring Harbor Symposium on Quantitative Biology,* 47 Pt 1, 189-95.

Seuri, M., Koivunen, J., Granfors, K. & Heinonen-Tanski, H. (2005) Work-related symptoms and Salmonella antibodies among wastewater treatment plant workers. *Epidemiology and Infection,* 133, 603-9.

SFS 2001:512. *Förordning (2001:512) om deponering av avfall.* Statens Författningssamling, uppdaterad 2005:424. http://62.95.69.3/SFSdoc /01/010512.PDF, 10-Aug-2006.

Sidhu, J., Gibbs, R. A., Ho, G. E. & Unkovich, I. (2001) The role of indigenous microorganisms in suppression of *Salmonella* regrowth in composted biosolids. *Water Research,* 35, 913-20.

Skiadas, I. V., Gavala, H. N., Lu, J. & Ahring, B. K. (2005) Thermal pre-treatment of primary and secondary sludge at 70 degrees C prior to anaerobic digestion. *Water Science and Technology,* 52, 161-6.

Smith, H. V. & Grimason, A.M. (2003) *Giardia* and *Cryptosporidium* In *The Handbook of Water and Wastewater Microbiology*. (Eds. D. Mara & N. Horan) Amsterdam, Academic Press, pp. 695-756.

Smith, S. R., Lang, N. L., Cheung, K. H. & Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Management,* 25, 417-25.

Solomon, E. B., Yaron, S. & Matthews, K. R. (2002) Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce

plant tissue and its subsequent internalization. *Applied and Environmental Microbiology,* 68, 397-400.

Stampi, S., De Luca, G., Varoli, O. & Zanetti, F. (1998/99) Occurrence, removal and seasonal variation of thermophilic Campylobacters and Arcobacter in sewage sludge. *Zentralblatt für Hygiene und Umweltmedizine,* 202, 19-27.

Statens Veterinärmedicinska Anstalt, National Veterinary Institute, Uppsala, Sweden. Tuberculos. http://www.sva.se/dok/611.html?searchstring=tuberculos&visaarkiv=1, 9-Aug-2006.

Steltzer, W., Jacob, J., Schulze, E. & Mochmann, H. (1991) Untersuchungen zum Vorkommen und Überleben von *Campylobacter* im Klärschlamm (A study of the occurrence and the survival of campylobacter in sludge). *Zentralblatt für Hygiene und Umweltmedizine,* 146, 17-23.

Sternberg, S. & Viske, D. (2003) Control strategies for paratuberculosis in Sweden. *Acta Veterinaria Scandinavica,* 44, 247-9.

Strachan, N. J., Fenlon, D. R. & Ogden, I. D. (2001) Modelling the vector pathway and infection of humans in an environmental outbreak of *Escherichia coli* O157. *FEMS Microbiology Letters,* 203, 69-73.

Strauch, D. (1991) Survival of pathogenic micro-organisms and parasites in excreta, manure and sewage sludge. *Review Scientific et Technique, Office International des Epizooties,* 10, 813-46.

Sugieda, M., Nagaoka, H., Kakishima, Y., Ohshita, T., Nakamura, S. & Nakajima, S. (1998) Detection of Norwalk-like virus genes in the caecum contents of pigs. *Archives of Virology,* 143, 1215-21.

SVARM 2005 (2006) Swedish Veterinary Antimicrobial Resistance Monitoring., The National Veterinary Institute (SVA), Uppsala, Sweden. www.sva.se, ISSN 1650-6332.

Swaminathan, B. (2001) *Listeria monocytogenes*. In *Food microbiology : fundamentals and frontiers.* (Eds. M. P. Doyle, L. R. Beuchat, & T. J. Montville)*,* Washington, DC, ASM Press, pp. 383-409.

SWEDRES 2005 (2006), A report on Swedish antibiotic utilisation and resistance in human medicine. Solna, Swedish Institute for Infectious Disease Control. www.strama.se, ISSN 1400-3473.

Talibart, R., Denis, M., Castillo, A., Cappelier, J. M. & Ermel, G. (2000) Survival and recovery of viable but noncultivable forms of Campylobacter in aqueous microcosm. *International Journal of Food Microbiology*, 55, 263-7.

Tenover, F. C., Arbeit, R. D., Goering, R. V., Mickelsen, P. A., Murray, B. E., Persing, D. H. & Swaminathan, B. (1995) Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology,* 33, 2233-9.

Thorn, J., Beijer, L. & Rylander, R. (2005) Hälsorisker till följd av exponering för mikroorganismer vid arbete i reningsverk - information till företagshälsovård. Health risks among sewage operatives in relation to microorganism exposure - information for industrial health service units *VA-Forsk rapport 2005-06,* Svenskt Vatten, Stockholm.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M. & Jennings, F. W. (1986) *Veterinary parasitology,* Longman Scientific & Technical, London, UK.

Wang, G., Zhao, T. & Doyle, M. P. (1996) Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Applied and Environmental Microbiology,* 62, 2567-70.

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

Wheeler, J., Sethi, D., Cowden, J. M., Wall, P. G., Rodrigues, L. C., Tompkins, D. S., Hudson, M. J. & Roderick, P. J. (1999) Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. *British Medical Journal,* 318, 1046-50.

WHO (2006) Risk to human health through potential transmission of avian influenza (H5N1) through water and sewage. WHO Document Production Services, Geneva, Switzerland.

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

Zabranska, J., Dohanyos, M., Jenicek, P., Ruzicikova, H. & Vranova, A. (2003) Efficiency of autothermal thermophilic aerobic digestion and thermophilic anaerobic digestion of municipal wastewater sludge in removing *Salmonella* spp. and indicator bacteria. *Water Science and Technology,* 47, 151-6.

Zabranska, J., Dohanyos, M., Jenicek, P., Zaplatilkova, P. & Kutil, J. (2002) The contribution of thermophilic anaerobic digestion to the stable operation of wastewater sludge treatment. *Water Science and Technology,* 46, 447-53.

Acknowledgements

Thanks to Swedish University of Agricultural Sciences (SLU), which funded this study through the research programme PROWARR (*Organic Waste - Resource or Risk in Sustainable Agriculture*).

I want to thank my supervisors: Karl-Erik, Kaggen, Johansson, my main supervisor, for his support and his competent advice, especially regarding bacteria; Ann Albihn, co-supervisor and project leader, for encouragement, enthusiasm and an unfailing thrust in me throughout the work with the dissertation; Anna Aspan. co-supervisor, for expertise, interest in my work and for support and push when it has been most needed, and Anders Gunnarsson, co-supervisor, for skilful help and positive encouragement.

Warm thanks to Marie-Louise Danielsson-Tham co-author and initial supervisor, for introducing me to the world of research; Anders Engvall, initial supervisor, for constructive criticism; Björn Bengtsson, co-author, for dedicated supervision regarding the issues of antimicrobial resistance; and Verena Rehbinder, co-author, for expert assistance, help and supervision at the antimicrobial laboratory.

Sincere thanks to my PhD-student colleagues and co-authors: Elisabeth Bagge, for close PhD-student fellowship and help with bacteria; Eva Emmoth, for good travel company and help with viruses; Annika Holmquist, for friendship and helping me with phages and helminths; and Birgitta de Jong, for stimulating discussions on epidemiology.

I also want to thank Ingela Berggren for constructive criticism of the manuscript, friendship, and for daily mental support; Annika Nordin, for helping me out with large and small, for friendship and good gossip; Mats Johansson, for collegial support and friendship during the first years; Jacob Ottosson and Björn Vinnerås, for creating a good and ambitious research environment.

Sofia Boqvist and Helene Wahlström, for wise comments on the manuscript.

Thanks also for laboratory assistance to AnnaLena Sahlin and other personnel at the bacteriology lab, Irene Dergel at the virology lab, and Bodil Christensson and Dan Christensson at the Department of Parasitology, and for technical assistance to Seved Helmersson at the food hygiene lab.

To Ulla Malmström, Eva Tysén, Carina Johansson, Helene Gustafsson and Ewa Backman for all technical and practical help during the years.

Many thanks to all other nice colleagues and friends at the department of Fish, Wildlife and Environment and the department of Disease Control and Biosecurity, for providing a welcoming and stimulating work environment.

Mary McAfee for checking the English.

And finally to the most important ones: Fritjof, Ellen, Anni and Albert, for always being there.