Ecology and modelling of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cattle manure and soil

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Abstract

The number of food poisoning cases caused by enteropathogens has increased in recent years. A significant part of the outbreaks associated with the consumption of raw vegetables has been attributed to *Escherichia coli* O157:H7 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Bovine manure and slurry are the main environmental sources of these pathogens. Thus, reduction of the multiplication of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in cattle and their survival in manure and slurry are important tasks to minimize the risks of contamination of plant products and outbreaks of food-borne diseases. This thesis describes the influence of various environmental factors on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure, slurry and soil amended with manure or slurry. Manure or slurry were inoculated with green fluorescent protein transformed strains of both enteropathogens at 10^6 - 10^7 cells g^{-1} dry weight, and their survival was studied in these substrates and in soil amended with inoculated manure or slurry. Population densities of the pathogens and autochthonous microbial communities were determined by dilution plating. The obtained survival data were fitted to non-linear models such as modified logistic or Weibull models, and estimated survival times in various substrates were compared. Analysis of the estimated parameter values showed that the pathogens survived longer at relatively low temperatures under anaerobic conditions especially at high concentrations of easily available substrate. *Salmonella* serovar Typhimurium was more resistant to environmental stresses than *E. coli* O157:H7. Survival of both pathogens significantly declined with increasing temperature amplitudes of daily temperature oscillations. Variations in fluctuations of *E. coli* O157:H7 populations around the decline curve were evaluated by the Approximate Entropy (ApEn) procedure. The instability of *E. coli* O157:H7 populations around the decline curve was greater in conventional than in organic and in loamy than in sandy soils, even though the mean survival periods did not differ. Multiple regression analysis of instability of *E. coli* O157:H7 survival on various soil characteristics showed a positive relation with the ratio of copiotrophic / oligotrophic bacteria, suggesting greater instability at higher available substrate concentrations. Percolation experiments with soil columns showed that surface application of solid manure decreased the risk of contamination of ground water and lettuce roots compared to injection of slurry, as more pathogen cells percolated to greater depths after slurry than after manure application. Detection of *E. coli* O157:H7 could be improved by incubation of Petri plates in anaerobic conditions, as this resulted in significantly higher numbers of recovered cells in comparison with the common aerobic plating procedure. Finally, a simulation model was developed based on our experimental data. The relative effects of temperature and substrate content were more important than that of oxygen concentration. The interaction with substrate resulted in oscillatory behavior of *E. coli* O157:H7 populations in manure and manure amended soil. Competition for substrate was the most important factor

affecting the final survival time. The model was used to evaluate the effects of various manure and soil management scenarios on the survival of *E. coli* O157:H7. This simulation model provides a new approach to investigate dynamic changes of invasive microorganisms in natural substrates. The results presented in this thesis can be used for risk assessment of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in dairy farming systems and will help to identify and evaluate potential control strategies to minimize the chance of pathogen spread in the vegetable production chain.

Contents

	Abstract	
Chapter 1.	General Introduction	1
Chapter 2.	Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* Serovar Typhimurium in cow manure	17
Chapter 3.	Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farmyard manure and slurry	37
Chapter 4.	Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils	61
Chapter 5.	Estimating the stability of *Escherichia coli* O157:H7 survival in manure amended soils with different management histories	91
Chapter 6.	Percolation and survival of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry	113
Chapter 7.	COLIWAVE a simulation model for survival of *E. coli* O157:H7 in dairy manure and manure-amended soil	139
Chapter 8.	General Discussion	173
	Summary	189
	Резюме	193
	Samenvatting	199
	Acknowledgements	205
	Curriculum Vitae	207
	Publication list	209
	Educational certificate of the Graduate School PE&RC	211

Chapter 1

General Introduction

Introduction

Consumption of milk and meat, including beef, has increased exponentially worldwide (Silbergeld et al., 2008; Sofos, 2008). An important side-effect of dairy and cattle farming is the production of manure and slurry (a mixture of manure and urine). Manure and slurry are usually applied to soil, fresh or after various periods of storage or composting. The main reason for soil application of manure or slurry is the enhancement of soil fertility. Soil fertility is primarily enhanced by mineral fertilizers on conventionally farmed land, but in areas with excess manure, organic fertilizers (manure or slurry) are used as well. On organically managed land, organic fertilizers are the main source of plant nutrition, as the use of synthetic fertilizers is generally not allowed.

Due to various negative side effects of the intensive use of mineral fertilizers (such as nitrate leaching, phosphate run-off, ammonia volatilization and nitrous oxide emission), mineral fertilizers are now partially replaced by organic fertilizers such as compost, treated and untreated manure or slurry. Usage of manure and other organic amendments is increasing world wide, especially in more sustainable farming systems in industrialized countries, but also in developing countries due to the high price of synthetic fertilizer. Utilization of farm-yard manure and slurry is the most economic and practical option for improving soil quality as well as providing an additional source of nutrients for growing plants. Moreover, organic fertilizers derived from manure have a relatively low negative impact on the environment. Both organic and conventional soils can be fertilized with liquid slurry and/or farm-yard manure. However, farm-yard manure is more frequently used at organic farms.

On the negative side, manure, especially untreated manure, may contain pathogenic organisms, in particular enteropathogenic bacteria. It has been estimated that food-borne diseases cause approx. 76 million illnesses, 325,000 hospitalizations and 5000 deaths each year in United States (Mead et al., 2000). A significant part of the outbreaks has been attributed to *Escherichia coli* O157:H7 and *Salmonella enterica* subsp *enterica* serovar Typhimurium (denoted as *Salmonella* serovar Typhimurium in this thesis). The annual cost of illness in the US due to *E. coli* O157:H7 alone was 405 million dollars in 2003 (Frenzen et al., 2005). In the USA and Canada, salmonellosis is economically the most important disease caused by *Salmonella* serovar Typhimurium followed by Shigatoxin - producing *E. coli* strains (primary *E. coli* O157:H7) (Mead et al., 1999). In Europe, the main enteric pathogens are *Campylobacter* and *Salmonella* (European Food Safety Authority, 2006), but *E. coli* O157:H7 infections are also increasing (Fisher and Meakins, 2006).

Farm animals are a major reservoir for these pathogens (Heuvelink et al., 1998; Boqvist and Vågsholm, 2005; Fossler et al., 2005; Hussein and Sakuma, 2005). Therefore, most attention has been paid to contamination of animal products. A large proportion of animal products can be contaminated with enteric pathogens. For

example, 36% of poultry samples were contaminated with *Salmonella* serovar Typhimurium in a Belgian retail market (Uyttendaele et al., 1999). Around 12% of minced beef samples in Spain, sampled between 1995 and 2003, contained Shigatoxin-producing *E. coli* including *E. coli* O157:H7 (Mora et al., 2007).

Substrates like manure and slurry have become a major concern with respect to food safety, since these substrates play an important role in the introduction of enteropathogens in the food chain (Natvig et al., 2002). However, it should be realized that enteropathogens can also cycle through the food chain and environment via soil, water, food and animal feed (Fig. 1). Transition of pathogens from manure through the food production chain has become more frequent in recent years (Natvig et al., 2002). Consequently, outbreaks of intestinal infections have increasingly been associated with bacterial pathogens ingested with vegetables and fruits (Beuchat, 1996; Sivapalasingam et al., 2004), especially in industrialized countries, but possibly also in developing countries, although exact figures are not known at a global level (Flint et al., 2005; Rangel et al., 2005). Contrary to animal products, which are generally cooked, various fruits and vegetables are consumed raw. Thus, many outbreaks have been associated with consuming raw produce, presumably contaminated from animal manure, water, or human handling (Michino et al., 1999; Tortorello and Reineke, 2000; Beuchat, 2002; Sivapalasingam et al., 2004; Rangel et al., 2005).

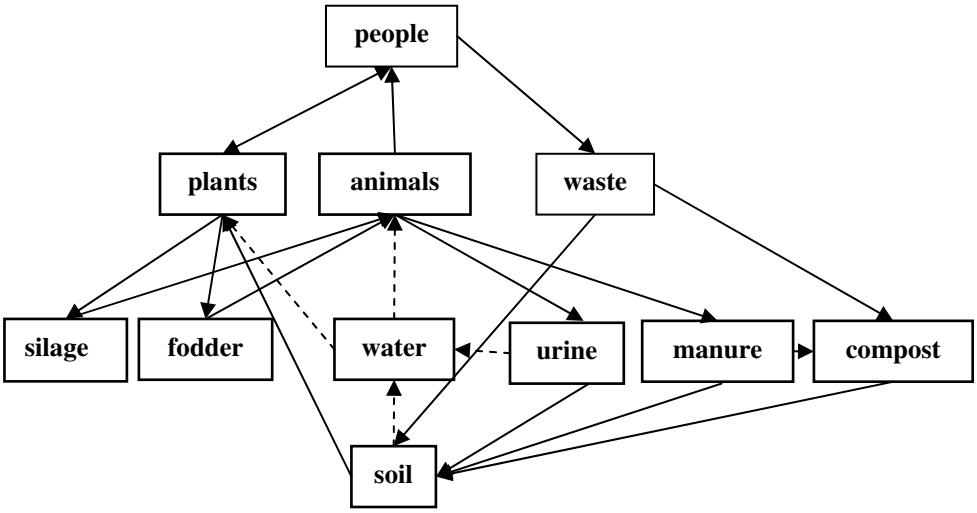

Fig. 1. Microbial cycle of enteropathogens.

Most outbreaks related to the consumption of fresh vegetables have been associated with *Salmonella* spp. and to a lesser extent with *E. coli* O157:H7 (Sivapalasingam et al., 2004). The best known examples are outbreaks of *E. coli*

O157:H7 from unpasteurized apple cider, lettuce or spinach, followed by salmonellosis associated with tomatoes, lettuce, melons and sprouts (Sivapalasingam et al., 2004). Recently, a large multi-state outbreak of *E. coli* O157:H7 associated with spinach occurred in the USA, with at least 187 cases, including 97 hospitalizations and three deaths (Anonymous, 2006). It has been shown that *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may become associated with the surface of plants growing in soil amended with contaminated manure (Natvig et al., 2002; Islam et al., 2004) and even be internalized into the plants (Solomon et al., 2002; Kutter et al., 2006; Franz et al., 2007; Klerks et al., 2007).

***Escherichia coli* and *Salmonella enterica:* biology and epidemiology**

E. coli bacteria are Gram-negative and belong to the gamma subclass of proteobacteria. *E. coli* cells are straight rods, motile by flagella or nonmotile. They are facultative anaerobic, having both a respiratory and a fermentative type of metabolism. Their optimum growth temperature is 37 ºC in pure culture. Verotoxigenic *E. coli* (VTEC) are strains of the bacterium *E. coli* capable of producing verotoxins, named for their toxicity towards Vero-cells, derived from kidney cells of particular monkeys. Verotoxins are also called shigatoxins, because they are very similar to toxins produced by *Shigella* species. Therefore, VTEC strains are also called STEC strains. Enterohemorrhagic *E. coli*, referred to as EHEC, are a part of the group of VTEC (or STEC) strains harboring additional pathogenicity factors besides these toxicity factors. Over 150 different serotypes of VTEC have been associated with human illness. The majority of reported outbreaks of VTEC infections are due to serotype O157. Symptoms caused by VTEC infections range from mild to bloody diarrhea, sometimes accompanied by fever. VTEC infection can also result in haemolytic uraemic syndrome (HUS). HUS is characterized by acute renal failure and anemia. HUS may develop in up to 10% of patients infected with VTEC O157. *E. coli* O157:H7 is very dangerous due to its resistance to low pH (pH 2.5), allowing unaffected passage through the stomach, and to its low infective dose (as few as 10 cells) and high pathogenicity (Tilden Jr et al., 1996).

Salmonella species are also Gram-negative bacteria with straight, motile rods. They belong to the gamma subclass of proteobacteria and are facultative anaerobic. Like for *E. coli*, their optimum temperature is 37 ºC in pure culture. The genus *Salmonella* is divided into two species: *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies. Most *Salmonella* strains isolated from mammals belong to the subspecies *S. enterica* subsp. *enterica*, including *S. enterica* subsp. *enterica* serovar Typhimurium (denoted as *Salmonella* serovar Typhimurium in this thesis). More than 2400 serovars of *Salmonella* exist and the prevalence of these different serovars is dependent on the host and environmental conditions. *Salmonella* has been known as an important enteropathogen of animals and humans. It is one of the most commonly

reported foodborne pathogens in the European Union and the USA. Human salmonellosis is characterized by fever, abdominal pain, nausea and vomiting. In the European Union *Salmonella* serovar Enteritidis and *Salmonella* serovar Typhimurium are the most frequent serotypes associated with human illness. *Salmonella* serovar Typhimurium is less pathogenic than *E. coli* O157:H7 but more widely spread in the world. Therefore, both of these pathogens are of great concern for public health (Beuchat, 1996; Joseph et al., 2002).

Farm animals normally carry these pathogens asymptomatically; i.e. they are carriers without showing the clear symptoms related to infections with these pathogens in humans. However, certain strains of *Salmonella* and *E. coli* can cause clinical disease symptoms in animals, leading to fever and diarrhoea (especially in calves and piglets). Both symptomatic and asymptomatic strains can be carried over via wildlife, including mammals and birds, to other animals or humans (Daniels et al., 2003). Poultry and pigs are the predominant reservoirs of *Salmonella* (Nicholson et al., 2005), while *E. coli* O157:H7 is mostly associated with cows. *E. coli* O157:H7 has also been found in deer (Renter et al., 2001) and flies (Alam and Zurek, 2004).

The prevalence of *E. coli* O157:H7 in dairy herds, as measured in manure, generally varies between 10% to 50% (Franz, 2007) or can be higher depending on the time of year, with a Shigatoxin gene prevalence as high as 80% of the manure samples (Franz, 2007). The prevalence of *Salmonella* serovar Typhimurium at swine herd and animal level was on average 59% and 17%, respectively, during the last 15 years (Funk and Gebreyes, 2004). The level of contamination of farm animals for both pathogens can be influenced by various factors, such as season, animal breed, age, housing, nutrition, antibiotic use, pathogen exposure, stress and on-farm hygiene (Brabban et al., 2004). Young animals are frequently more susceptible to pathogens, including human pathogens, than older ones. Higher cattle densities and increased cattle movements may contribute to the spread of *E. coli* O157:H7 (Vanselow et al., 2005). The effect of feed composition on the prevalence of *E. coli* O157:H7 in cow intestines and manure has been well documented (Russell and Rychlik, 2001). Feeding cows with high carbohydrate diets such as maize silage or corn results in a higher prevalence of *E. coli* O157:H7 in manure than feed that contains older grass silage, hay or straw (Franz et al., 2005). Only a slight influence of cattle diet was found on the prevalence of *Salmonella* serovar Typhimurium in manure in the same study (Franz et al., 2005). Cattle fed with large amounts of raw corn had approximately 1000-fold higher numbers of *E. coli* cells than cattle fed with hay (Diez-Gonzalez et al., 1998). Thus, animal diet seems to be a major factor influencing the prevalence of enteropathogens in the animals and their manure.

Animal faeces and irrigation water are the main avenues for the spread of human pathogens to fields and the crops growing there (Solomon et al., 2002; Islam et al., 2004). Bovine manure and slurry are main environmental sources of *E. coli*

O157:H7 and *Salmonella* serovar Typhimurium with average densities between 10^3 and 10^4 CFU gdw^{-1} of manure or slurry (Nicholson et al., 2005), but the density can be as high as 10^7 CFU gdw^{-1} of manure (Fukushima and Seki, 2004). These high densities can persist for periods of time ranging from several days to almost a year (Kudva et al., 1998). The survival period in soil after application of contaminated manure may depend on soil management practices (e.g. organic versus conventional), manure type and method used for application, available substrate in relation to microbial competition, bacterial diversity, temperature, moisture, and presence of oxygen (Franz, 2007).

Human enteropathogens in manure, slurry or irrigation water invade vegetable and other arable crop plants after application of organic fertilizers to soils. These pathogens may adhere to roots or contaminate leaf surfaces by splashing that occurs during rainfall or irrigation (Natvig et al., 2002). Internal plant compartments may be colonized (Solomon et al., 2002; Klerks et al., 2007), so that these pathogens can not be easily washed from consumable plant parts or killed by disinfectants anymore. From infected consumable products, enteropathogens can further spread to unspoiled products during food processing and packaging, resulting in a wider spread of enteropathogens to different products made in the food production chain (Guo et al., 2001).

Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and soil

The level of contamination of plants and their consumable products with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium will largely depend on survival of these pathogens in different environments like manure, slurry and in soil amended with these organic fertilizers. Although conditions for survival of enteric pathogens in manure are considered to be less favourable than in the animal gut system (Tauxe et al., 1997), *E. coli* O157:H7 and *Salmonella* serovar Typhimurium can survive for extended periods of time ranging from several days to almost a year (Kudva et al., 1998; Fukushima et al., 1999; Jiang et al., 2002; Nicholson et al., 2005). In the past, animal manure was stored or composted for several months, reaching conditions unsuitable for enteropathogen survival (temperatures of 55 ºC or higher) (Nicholson et al., 2005). With the advent of intensive farming, use of manure and slurry changed fundamentally to the application of raw (untreated), instead of long-term stored or composted faeces in many parts of the world. Due to the increases in enteritidis outbreaks, the use of fresh manure and slurry has been regulated by law in many countries to reduce possible risk of contamination. For example, composting of manure is nowadays mandatory for organic farming systems in the USA before it can be applied to cropland (U.S. Department of Agriculture, 2000). In other parts of the world these regulations are not in effect and raw manure is still applied in vegetable

production. Thus, preventing accumulation of the pathogens in cattle and reducing their survival in faeces are important tasks for reducing the risk of food-borne diseases (Franz et al., 2005).

The effects of chemical composition of manure and soil amended with manure on human enteropathogens has been investigated (Franz et al., 2005; Franz et al., 2008) and factors like, moisture content and oxygen status (Kudva et al., 1998), pH (Himathongkham et al., 1999), and temperature (Wang et al., 1996; Kudva et al., 1998; Himathongkham et al., 1999; Wang et al., 2004; Nicholson et al., 2005) were shown to be important too. Different combinations of all of these factors lead to differences in survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure or soil.

Temperature is one of the most critical factors posing strong effects on chemical composition and autochthonous microbial community structures and on survival of enteropathogens in manure and soil. Survival time of enteropathogens in bovine manure was extended from several days at a temperature of 37 ºC to several months at a temperature of 4 ºC (Wang et al., 1996; Kudva et al., 1998; Himathongkham et al., 1999), although the optimal temperature for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium under laboratory conditions in broth is 37 ºC (Minor, 1984; Orskov, 1984). *Salmonella* serovar Typhimurium generally survives for longer periods of time in manure under various temperatures than *E. coli* O157:H7 (Himathongkham et al., 1999). Similar survival periods for the pathogens were obtained for soils or soil amended with manure (Vinten et al., 2002; Franz et al., 2005). In general, the decline rates of both pathogens increase at temperatures ranging from -20 to 70 ºC in natural substrates (Kudva et al., 1998; Himathongkham et al., 1999). Most likely temperatures affect the activity and composition of the autochthonous microbial communities in manure and soil, which exert an indirect effect on enteropathogen survival. In addition, the effects of temperature on survival of enteropathogens is likely to be different under oscillating than under constant temperatures (Scherm and van Bruggen, 1994). Surface temperatures of a manure pile or manure amended soil can be subject to considerable diurnal oscillations, but the effects of oscillating versus constant temperatures on survival of enteropathogens has not been investigated.

The presence or absence of oxygen in manure, slurry or soil may also lead to differences in survival time of enteropathogens. The behaviour of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium cannot be easily predicted since both pathogens are facultative anaerobic bacteria and are able to use aerobic and anaerobic types of metabolism in different oxygen conditions. Thus, *E. coli* O157:H7 survived for more than 1 year in non-aerated (anaerobic) ovine manure, while in corresponding aerated conditions survival time was not more than 4 months (Kudva et al., 1998). In another study, *E. coli* O157:H7 was still detected after 42 days in turned bovine manure heaps whereas in unturned manure *E. coli* O157:H7 was still detectable after 90 days

(Fremaux et al., 2007). An accurate relationship between survival time of *Salmonella* serovar Typhimurium and presence of oxygen has not been quantitatively investigated. It is important to establish this relationship since oxygen depletion likely occurs under all circumstances in manure heaps, and survival time of human enteropathogens in manure may be extended in the absence of oxygen.

In several experiments survival of enteropathogens was related to pH reflecting the presence of acidic compounds in soil or manure. It is known that both *E. coli* O157:H7 and some *Salmonella* strains possess systems for surviving at low pH and therefore can be considered as acid resistant bacteria (Foster and Spector, 1995; Lin et al., 1996). Survival time of *E. coli* O157:H7 was shorter in manure at a relatively high pH than at lower pH levels (Bach et al., 2005; Franz et al., 2005). Manure pH is largely determined by cattle diet. Survival of *E. coli* O157:H7 was shorter in manure derived from a straw diet in comparison with survival in manure from a high-digestive grass silage plus maize silage diet (Franz et al., 2005). Type of the diet had only a small influence on *Salmonella* serovar Typhimurium survival time (Franz et al., 2005). Generally, *E. coli* O157:H7 survives longer in anaerobic conditions at low temperature, low pH and high levels of dissolved organic carbon (DOC) (Franz et al., 2005). For *Salmonella* serovar Typhimurium, temperature is the main factor affecting survival time in manure and soil amended with manure, while survival of this species is less affected by oxygen concentration, pH and DOC.

The pathogens interact with the rest of the microbial community in the intestinal tract, manure and soil. However, the conditions differ in these substrates, so that competition with the microbial community will change. Likely, the impact of the microbial competition on survival of enteric pathogens will increase as the substrate is farther removed from the reservoir of these pathogens. The influence of the microbial community was clearly shown in experiments where *E. coli* O157:H7 survived significantly longer in manure-amended autoclaved soil than in manure-amended non-autoclaved soil at 15 ºC (Jiang et al., 2002). However, autoclaved soil is barely comparable with fresh soil in terms of physical or chemical processes which take place in this substrate. The influence of autochthonous microbial communities on pathogen survival and spread has hardly been explored to date due to the fact that diversity and abundance of microbial communities could not be characterised easily in the past. At present, microbial composition, diversity and abundance as well as short term changes of these characteristics can be monitored by application of molecular fingerprinting techniques (Ibekwe and Grieve, 2004). With these techniques significant shifts in the autochthonous microbial community structure in manure was demonstrated after storage (Leung and Topp, 2001; Snell-Castro et al., 2005), indicating that survival times of enteropathogens may be affected by changes in microbial community structures during storage of manure.

Soil management practices and soil organic matter composition have a significant influence on the microbial community (van Diepeningen et al., 2006; Fließbach et al., 2007) and therefore have an indirect effect on spread and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Conventional farming systems are characterized by intensive farming procedures, application of artificial fertilizers, pesticides and antibiotics, which presumably result in a decrease of bacterial diversity, reduction of soil organic matter and microbial biomass in comparison with organically managed systems (Giller et al., 1998; Mader et al., 2002; van Diepeningen et al., 2006). Further, the survival time and spread of *E. coli* O157:H7 is affected by the different ways manure or slurry is stored and applied to farm land (Nicholson et al., 2005). Under various management practices the percolation of enteropathogens through soil layers may be different (Gagliardi and Karns, 2000). However, the movement and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in different soil layers after application of manure and slurry is still insufficiently quantified.

Most experiments done on survival of enteropathogens in manure, slurry, soil and on (in) plants focused on survival times of the enteropathogens without paying much attention to the factors that influence human enteropathogen survival (McClure & Hall, 2000). In these experiments the combined influence of abiotic and biotic factors on spread and survival of enteropathogens in natural substrates was not checked systematically in a quantitative manner. Only the influence of independent environmental characteristics on enteropathogen survival has been investigated in some natural substrates. Still very little is known about the influence of multiple factors naturally existing in manure and soil on enteropathogen survival, such as the chemical composition, structure of autochthonous microbial communities, fluctuation in temperature and oxygen levels, or farm management (Guan & Holley, 2003). Since both spread and survival of the enteropathogens likely depend on interactions between the various environmental factors, the overall set of abiotic and biotic environmental characteristics should be taken into account. As it is difficult to do this in multifactorial experiments, it would be beneficial to have a simulation model where the effects of many factors on pathogen survival can be studied simultaneously. Such a simulation model is currently not available, since all previous models were mainly empirical, based on mathematical relations between biotic and abiotic factors and growth or survival of certain microorganisms (Gibson et al., 1987; Zwietering et al., 1990; Whiting and Cygnarowicz-Provost, 1992).

Scope of this thesis

There are two main objectives in the thesis: 1) to identify factors that minimize pathogen spread, multiplication and survival in primary plant production chains where manure is applied for fertilization of soil and 2) to develop a simulation model for prediction of enteropathogen survival in manure and soil amended with manure under

different management scenarios. These objectives have been addressed in 8 chapters (Fig 2):

Chapter 1 provides an overview of recent studies about the epidemiology and ecology of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, and the influence of physical, chemical and biological characteristics of manure, slurry and soil on survival of these enteropathogens.

Chapter 2 presents the effect of static and fluctuating temperatures on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure with and without the presence of the autochthonous microbial community

Chapter 3 describes the influence of aerobic and anaerobic conditions on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and explores the sensitivity of two plating methods for detection of the enteropathogens

Chapter 4 provides the results of extensive research on the influence of soil type and management, biotic and abiotic factors on survival of *E. coli* O157:H7 in soil amended with contaminated manure

Chapter 5 compares the stability of *E. coli* O157:H7 populations in different soils amended with manure in relation to soil management and various biological and chemical characteristics of the manure amended soils

Chapter 6 describes the influence of application methods of manure and slurry to soil, and the presence of the rhizosphere of lettuce plants on translocation of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium through the soil as well as their survival at different depths in the soil profile

Chapter 7 presents a simulation model of *E. coli* O157:H7 dynamics in manure and manure amended soil and the results of simulation experiments to assess the transmission of this pathogen through part of the primary production chain (manure – soil) and to calculate the efficiency of different scenarios of manure storage and application in controlling *E. coli* O157:H7

Chapter 8 finalizes the thesis by discussing the obtained results and their contributions to a better understanding of both the risk of contamination with enteropathogens as well as their ecology during spread and survival.

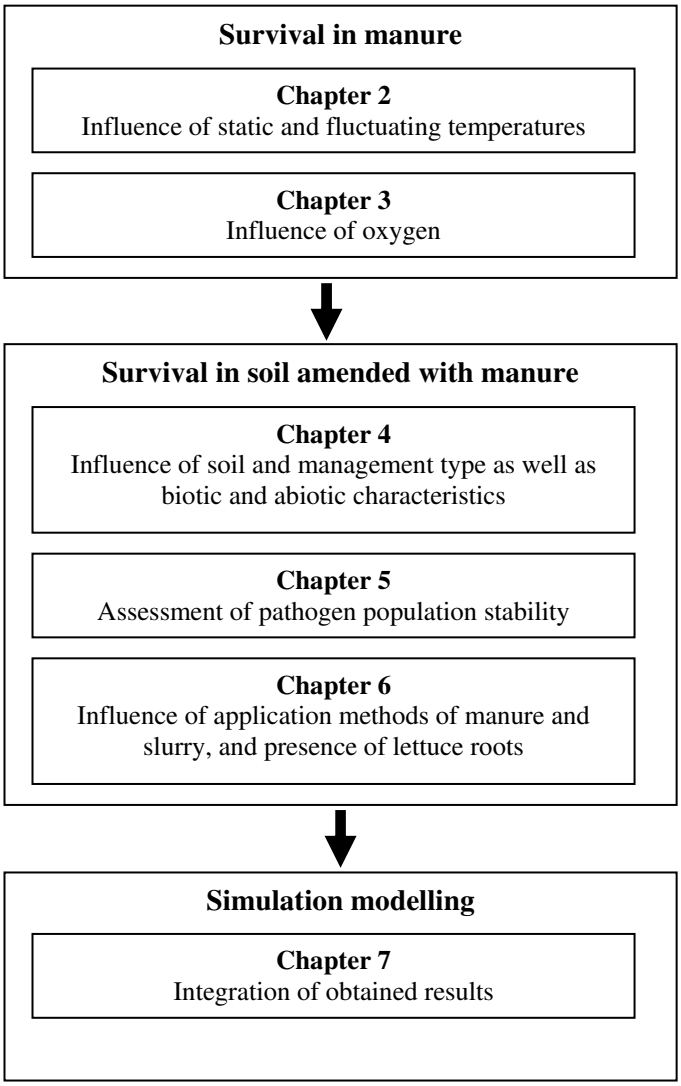

Fig. 2. Thesis outline.

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Chapter 2

Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure

Semenov, A.V., van Bruggen, A.H.C., van Overbeek, L., Termorshuizen, A.J., and Semenov, A.M. (2007) Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in cow manure. *FEMS Microbiology Ecology* **60**: 419-428.

Abstract

Effects of four average temperatures (7, 16, 23 and 33 °C) and daily oscillations with three amplitudes (0, ±4, ±7 °C) on survival of the enteropathogens *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium were investigated in small microcosms. Manure was inoculated with a green fluorescent protein transformed strain of either pathogen at 10^7 cells/gram of dry weight. Samples were collected immediately after inoculation, and one and two weeks after inoculation for *E. coli* O157:H7, and immediately and after two and three weeks for *Salmonella* serovar Typhimurium. Population densities were determined by dilution plating and direct counting. In addition, total bacterial CFUs were determined. Growth and survival data were fitted to a modified logistic model. Analysis of the estimated parameter values showed that *E. coli* O157:H7 survived for shorter periods of time and was more sensitive to competition by the native microbial community than *Salmonella* serovar Typhimurium. Survival of both pathogens significantly declined with increasing mean temperatures and with increasing amplitude in daily temperature oscillations. The results indicated that responses of enteropathogens to fluctuating temperatures cannot be deduced from temperature relationships determined under constant temperatures.

Introduction

The incidence of gastroenteritis and food poisoning caused by fecal pathogens being transmitted via fresh produce has increased in industrialized countries over the last 20 years, and possibly in developing countries, although exact figures are not known at a global level (Flint *et al.*, 2005; Rangel *et al.*, 2005). The annual cost of illness in the US due to *E. coli* O157:H7 alone was 405 million dollars in 2003 (Frenzen *et al.*, 2005). Many of the outbreaks of intestinal infections and deaths have been associated with consuming uncooked vegetables and fruits, presumably contaminated from animal manure, water, or human handling (Beuchat, 2002; Rangel *et al.*, 2005). *E. coli* O157:H7 is very dangerous due to its low infective dose (as few as 10 cells) and high pathogenicity (Tilden Jr *et al.*, 1996). *Salmonella* serovar Typhimurium is less pathogenic but widely spread in the world. Therefore, both these pathogens are of great public concern (Beuchat, 1996; Joseph *et al.*, 2002).

Cattle are a major reservoir for these pathogens (Boqvist and Vågsholm, 2005; Fossler et al., 2005; Hussein and Sakuma, 2005). Thus, preventing their accumulation in cattle and reducing their survival in feces form important avenues for reducing the risk of contamination of plant products and reducing the risk of food-borne diseases (Franz *et al.*, 2005). Survival and spread of enteropathogens in the agricultural production chain is greatly affected by the way manure is handled, stored, and applied (Kudva *et al.*, 1998; Nicholson *et al.*, 2005). In the past, animal manure was stored or composted for several months, commonly reaching temperatures greater than 55 ºC (Nicholson *et al.*, 2005). With the advent of intensive farming, manure treatment and use changed fundamentally to the application of raw manure and slurry in many parts of the world. Due to the recent increase in enteritidis outbreaks, composting of manure became mandatory in the USA before it could be applied to cropland, however only for organic farms (U.S. Department of Agriculture, 2000). In other parts of the world, raw manure is still applied.

Various manure characteristics, such as chemical composition (Franz *et al.*, 2005), moisture content as well as environmental factors during storage such as oxygenation (Kudva *et al.*, 1998), pH (Himathongkham *et al.*, 1999), and temperature (Wang *et al.*, 1996; Kudva *et al.*, 1998; Himathongkham *et al.*, 1999; Wang *et al.*, 2004) lead to differences in survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure. Temperature is one of the most important factors, having a large effect on both the intensity of chemical reactions and growth and death rates of the native microorganisms, and undoubtedly also on enteropathogens in manure. *E. coli* O157:H7 survived from several days at 37 ºC to several months at 4 ºC in bovine manure (Wang *et al.*, 1996; Kudva *et al.*, 1998; Himathongkham *et al.*, 1999). *Salmonella* serovar Typhimurium generally survives for longer periods of time in manure under various temperatures than *E. coli* O157:H7 (Himathongkham *et al.*, 1999). In general, the decline of both pathogens increases with temperature in natural

substrates such as soil, manure and slurry at temperatures ranging from -20 to 70 ºC (Kudva *et al.*, 1998; Himathongkham *et al.*, 1999), although the optimal temperature for these enteropathogens under laboratory conditions in broth is about 37 ºC (Minor, 1984; Orskov, 1984).

The microbial community also has a great impact on survival of enteric pathogens. *E. coli* O157:H7 survived significantly longer in manure-amended autoclaved soil than in manure-amended non-autoclaved soil at 15 ºC (Jiang *et al.*, 2002). At different temperatures the autochthonous microbial community would probably change and exert a different influence on enteropathogen survival.

Previous experiments on survival of enteropathogens were commonly carried out under static environmental conditions (temperature, moisture content etc.), but in reality, the physical conditions and microbial community composition change continuously, with a diurnal circadian rhythm. Effects of constant temperature conditions on growth and survival of pathogens have generally been used in predictive models for risk assessment (Bovill *et al.*, 2001). Yet, it is already well known from other areas in biology, that effects of oscillating temperatures with certain mean temperatures can be very different from constant temperature effects with the same mean temperatures (Scherm and van Bruggen, 1994; Fantinou et al., 2003). Moreover, frequent fluctuation of ambient temperature around freezing caused more rapid bacterial death (Natvig *et al.*, 2002).

Very few controlled experiments have been carried out to investigate effects of fluctuating temperatures on microbial dynamics, including food-borne pathogens. It was shown only recently that *E. coli* behaves differently in nutrient broth under fluctuating compared to constant temperatures (Jones *et al.*, 2004). Nevertheless, there is no information about the behavior of human pathogens in natural substrates such as manure under diurnal, oscillating temperature conditions. Furthermore, several studies indicated a more complex behavior of bacterial populations in natural substrates (Zelenev *et al.*, 2005) than was assumed before. It is important to understand the dynamics of enteric pathogens in natural substrates to predict the risk of exposure to these pathogens through agricultural products.

In the present study we simulated storage of manure in small microcosms under dynamic temperature conditions close to reality, with and without the influence of the native microbial community. The objectives were: to determine the effects of various mean temperatures and temperature amplitudes on growth and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium at weekly intervals in sterilized and non-sterilized manure and to investigate the effect of competition and/or antagonism from the autochthonous microbial community.

Materials and Methods

Bacteria. *E. coli* O157:H7 strain B6-914 *gfp*-91 was provided by Pina M. Fratamico (Fratamico *et al.*, 1997). The strain had been modified from strain SEA 13B88 (from the outbreak linked to Odwalla apple cider; Food and Drug Administration), so that it contained green fluorescent protein (*gfp*) (pGFO cDNA vector) and ampicillin resistance, while the Shiga-like toxins ($Stx1^-$ and $Stx2^-$) were deleted. These changes did not result in any significant differences in survival in nutrient media compared to the wild-type strain (Fratamico *et al.*, 1997). *Salmonella* serovar Typhimurium MAE 119 (ΔagfD101 *saw*) was obtained from Ute Römling (Römling *et al.*, 1998; Römling *et al.*, 2000). This strain carried resistance to kanamycin and gentamycin and carried the *gfp* gene after transformation with the PAG408 mini-transposon. No differences between the wild-type of *Salmonella* serovar Typhimurium and its transformed form were found (Römling *et al.*, 2000). Green fluorescence of both *gfp*-transformed strains was checked under UV light. Stock cultures were stored in 30% (w/w) glycerol at -80°C.

Manure. Fresh manure without urine from organically managed Holstein Frisian steers on a standard 50% grass/clover-silage + 50% dried grass diet was mixed with straw (90% manure and 10% straw (kg/kg, dry weight) and stored (50-60 ^oC at 20 cm depth) for nearly one month in a heap at the organic experimental farm Droevendaal (Wageningen University and Research Center, The Netherlands). About 10 kg of this manure was collected from the heap in February 2005, homogenized and stored in closed plastic bags at 5 °C for two weeks. To obtain sterilized manure for some experiments, a plastic, hermetically sealed jar with manure was gamma-irradiated with 39.6 kGy from Co_{60} (Isotron, Ede, the Netherlands) for one day. The sterile condition of the manure was checked by dilution-plating of approximately 3 g of gamma-irradiated manure on Luria-Bertani medium, and incubated for 48 h. The dry weight of both natural and sterilized manure was determined after heating for 24 h at 105 °C. Various chemical characteristics were determined at the beginning of each experiment (Table 1). The pH of manure samples was measured in water suspension 1:2.5 (g/v). The pH and dry matter were also measured at the end of the experiments. No significant changes in water content and pH were observed. Total carbon was determined by CHN1110 analyzer (CE Instruments, Milan, Italy) using the Dumas method (Suehara *et al.*, 2001). Total nitrogen was analyzed by the Kjeldahl method (Kemsley *et al.*, 2001). Ammonium was determined in trichloroacetic acid solution by Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, NY, USA).

Inoculation of manure. Bacterial inocula were grown in Erlenmeyer flasks containing 150 ml fresh Luria-Bertani broth, with 50 μg/ml ampicillin for *E. coli* O157:H7 and 50 μg/ml kanamycin (both, Sigma-Aldrich Chemie Gmbh, Germany) for *Salmonella*

Table 1. Chemical analysis of untreated and sterilized manure, used in the temperature experiments

Manure	pH	Dry matter (g/kg)	N-NH_4 (g/kg)	Total N (g/kg)	Total C (g/kg)	C/N
Untreated	8.1	193.5	0.99	18.3	420.7	22.99
Sterilized	8.2	167.3	1.45	20.1	400.1	19.90

Table 2. Estimated time needed to reach the detection limit of 1 log CFU gdw^{-1} for *E. coli* O157:H7 in untreated manure under different dynamic temperature conditions

Survival time ± SD (days)			
Mean temperature, °C	Daily amplitude, °C		
	± 0 °C	± 4 °C	± 7 °C
7 °C	159.4 ± 77.2	99.1 ± 6.8	89.4 ± 34.1
16 °C	57.4 ± 2.3	51.4 ± 1.8	46.2 ± 1.7
23 °C	35.7 ± 0.3	32 ± 1.5	30 ± 0.5
33 °C	<7	<7	<7

Table 3. Estimated time needed to reach the detection limit of 1 log CFU gdw^{-1} for *Salmonella* serovar Typhimurium in untreated manure under different dynamic temperature conditions

	Survival time ± SD (days)		
Mean temperature, °C	Daily amplitude, °C		
	± 0 °C	± 4 °C	± 7 °C
7 °C	227.3 ± 38.3	184.5 ± 38.7	131.7 ± 19.9
16 °C	74.9 ± 4.7	67.9 ± 4.2	63.1 ± 1.9
23 °C	47.5 ± 4.5	44.3 ± 2.7	43.8 ± 3.2
33 °C	<21	<21	<14

serovar Typhimurium, followed by incubation at 37°C on an orbital shaker (200 rev min^{-1}) for 18 h to reach the stationary phase of cells. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The cell density of the suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm in 1 ml cuvet would equal approximately 1×10^9 CFU ml^{-1}. Prepared inocula were added by a pipette to manure to a final density of 10^8 CFU per gram of manure dry weight (gdw^{-1}) and mixed thoroughly within a double layer of plastic autoclavable bags. After thorough kneading of plastic bags by hand for 5 min., the inoculated manure (around 23 g) was transferred to Petri plates (diam. 52 mm). The Petri plates were closed by PetriSEAL (DiversifiedBiotech, USA), which provides air exchange and prevents moisture loss. To prevent possible contamination with genetically modified bacteria, each small plate was put into a bigger one (diam. 86 mm) and closed by PetriSEAL as well.

Setup of experiments. Four different experiments were carried out in sterilized and non-sterilized manure to establish the influence of static versus fluctuating temperatures on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Experiments were done separately for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, and separately with untreated and sterilized manure. For all experiments, manure of the same origin was used. Twelve treatments (four mean temperatures: 7, 16, 23 and 33 °C with 3 amplitudes (±4 and ±7°C and 0 °C as control) were chosen for the experiments with untreated manure and eight for experiments with sterilized manure (four mean temperatures: 7, 16, 23 and 33 °C with 2 amplitudes: ±7 °C and 0 °C as control, respectively). Selection of the temperature treatments for the experiments was based on seasonal temperature variations in soil (Stoller and Wax, 1973) and manure (Nicholson *et al.*, 2005). Three replicates were randomly selected for destructive sampling at each of three time points per treatment, immediately after inoculation, after one and two weeks for *E. coli* O157:H7, or after two and three weeks for *Salmonella* serovar Typhimurium.

Temperature control. A special temperature table with 100 individual cells (designed by IMAG, Wageningen University and Research Center, the Netherlands) was used. The temperature was computer-controlled by heating or cooling an aluminum container inside each individual cell with high accuracy and flexibility. Temperature fluctuations were programmed for each cell individually to have sine waves with one period per day. Each temperature treatment was started at 18 °C and during the next 12 h reached its mean temperature level (7, 16, 23 or 33 °C) in order to prevent a sudden temperature change at the start of each experiment. Each cell contained one Petri dish with inoculated manure. There were 2 control plates with non-inoculated manure per experiment, maintained at constant temperatures (7, 16, 23 or 33 °C).

Sampling procedure of manure. Samples of manure, approximately 0.5 g, were put in pre-weighed dilution tubes with 4.5 ml of sterile distilled water to determine the exact weight. Samples were vortexed and sonicated for 30 sec (Branson 5200, 120-W output power, 47 kHz). Ten-fold dilution series were prepared with sterile distilled water, and 50 µl of the two highest dilutions per sample was plated in duplicate on sorbitol-MacConkey agar (Oxoid) with ampicillin (50 µg/ml) for *E. coli* O157:H7 and on Luria-Bertani agar with kanamycin (50 µg/ml) for enumeration of *Salmonella* serovar Typhimurium, respectively. After adding approximately 20 sterile glass beads per Petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass beads to spread the inoculum over the surface of the plate. Fluorescent bacterial colonies were counted under a UV lamp (365 nm UV-A, PL-S, Philips, Eindhoven, the Netherlands) after incubation at 37 °C for 24 h. Fluorescent colonies made up 95-99% of all colonies on a plate. Fluorescent colony-forming units (CFU) were calculated per gram of dry manure.

To determine the density of total cultivable bacteria in experiments with inoculated untreated manure, dilution series were prepared as described above. Fifty µl of each sample was plated in duplicate on LB medium and incubated at 37 °C for 24 h. All colonies with typical bacterial morphology were counted.

Direct microscopic counts of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were determined for fresh manure only. Microscopic slides were prepared with 10 µl of 10^{-1} and 10^{-2} dilutions. Approximately 100 fields were observed under an epi-fluorescent microscope (ZEISS 'Axioscop' 20 with a HBO 50 mercury lamp for fluorescence-illumination, Carl Zeiss Jena GmbH, D-07740 Jena, Germany). A blue UV light filter (450-490 nm) was used for *gfp* visualization (Luby-Phelps *et al.*, 2003; Oda *et al.*, 2004). Possible fluorescent background was checked in suspensions of manure from control tubes without inoculation with *gfp*-containing bacteria. The level of confidence for direct microscopy is 8.0×10^5 cells gdw^{-1}.

Statistical analysis. First, number of colonies for each Petri plate was calculated to CFU gdw^{-1} and standard deviations were calculated for every temperature treatment. ANOVA tests were done for CFUs and direct cell counts per g of dry manure after two weeks, to assess the influence of temperature and its fluctuations on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium using the GLM procedure of the SAS program (SAS Institute Inc., Cary, USA). To describe the decline in CFUs and direct counts over time in non-sterilized manure, log-transformed data were fitted to a modified logistic function by nonlinear regression (Gauss-Newton method): $C_t = a / (1 + c \times e^{(-m \times t)})$, where C_t is the log CFU gdw^{-1} at time t (days), a is the upper asymptote (CFU gdw^{-1}), c is a parameter for the shoulder (days), and m is a slope parameter for the rate of change (days^{-1}). For declining populations m is negative, while for increasing populations it is positive. This model was selected because we previously

showed excellent fits of data on decline of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in similar cattle manure, where 6 samplings were done during the first 25 days of the survival experiment (Franz *et al.*, 2005). The parameters for the survival curves under different temperature conditions were estimated with the NLIN procedure of the SAS program (SAS Institute Inc., Cary, USA). The significance and fit of the estimated decline rates were assessed by the F value of the non-linear regression and the non-linear coefficient of determination (pseudo-r^2) for each curve, respectively. Growth and decline of *Salmonella* serovar Typhimurium in sterilized manure was analyzed by fitting of survival data to the exponential function $C_t = N_0 \times e^{(-m \times t)}$, where C_t is the log CFU gdw^{-1} at time t (days), N_0 is initial log CFU gdw^{-1} on day 0 and m is a slope parameter. The effects of mean temperature, amplitude of temperature fluctuation and sterilization treatment on rates of change (days^{-1}) were analyzed with ANOVA using the General Linear Models (GLM) procedure.

Results

Influence of static and oscillating temperatures on *E. coli* O157:H7 in manure. Immediately after inoculation, the density of *E. coli* O157:H7 was 7.68 ± 0.08 log CFU gdw^{-1} of untreated manure. Pathogen populations significantly declined during the two-week incubation period in fresh manure at each of the temperature treatments (Fig. 1a). At a constant temperature of 7 ºC, CFUs decreased slightly but significantly ($p<0.05$) to 7.44 ± 0.10 log CFU gdw^{-1} after two weeks. At 7 ± 4 and 7 ± 7 ºC, there were slightly fewer CFUs remaining after 14 days than at a constant temperature of 7 ºC, but the differences between oscillating and constant temperatures were not significant. Treatments with a mean temperature of 16 ºC resulted in a more rapid decrease in CFUs than those at 7 ºC, the final densities ranging from 6.96 ± 0.05 log CFU gdw^{-1} for the static variant to 6.85 ± 0.12 and 6.63 ± 0.12 log CFU gdw^{-1} for 16 ± 4 and 16 ± 7 ºC, respectively. Final density at 16 ± 7 ºC was significantly lower than at constant temperature ($p<0.05$). The decline in CFUs of *E. coli* O157:H7 was greater in manure stored at constant 23 ºC ($p<0.001$), where log CFUs decreased to 6.09 ± 0.05 gdw^{-1} after 2 weeks. The effects of temperature oscillations were more pronounced at a mean temperature of 23 ºC than at lower mean temperatures ($p<0.05$). At 23 ± 4 and 23 ± 7 ºC, the final densities dropped to 5.67 ± 0.27 and 5.43 ± 0.16 log CFU gdw^{-1}, respectively. Finally, at 33 ºC, *E. coli* O157:H7 declined so quickly, that already after one week it could not be detected by plate counting, both after exposure to constant and oscillating temperatures.

Changes in *E. coli* O157:H7 densities during the experimental period were very different in sterilized compared to untreated manure (Fig. 1c). Immediately after inoculation, the population density was 8.23 ± 0.13 log CFU gdw^{-1}. After two weeks at an average temperature of 7 ºC, there was a slight drop to 7.84 ± 0.11 regardless of temperature oscillation (at 7 ± 7 ºC the final density was 7.79 ± 0.09 log CFU gdw^{-1}).

Manure samples exposed to 16 and 23 ºC showed significant ($p<0.05$) growth in all cases, final densities reaching 9. 27 ± 0.08 and 9.05 ± 0.09 log CFU gdw^{-1} at 16 and 16 ± 7 ºC, and 9.05 ± 0.12 and 8.32 ± 0.05 log CFU gdw^{-1} at 23 and 23 ± 7 ºC, respectively. Contrary to the non-sterilized manure, *E. coli* O157:H7 survived at 33 °C although it did not increase initially as it did at 16 and 23 °C. Oscillating temperatures at 33 ± 7 °C resulted in a decline to 6.46 ± 0.25 log CFU gdw^{-1} compared to 7.65 ± 0.20 log CFU gdw^{-1} at constant 33 ºC. At all mean temperatures, except 7 ºC, the

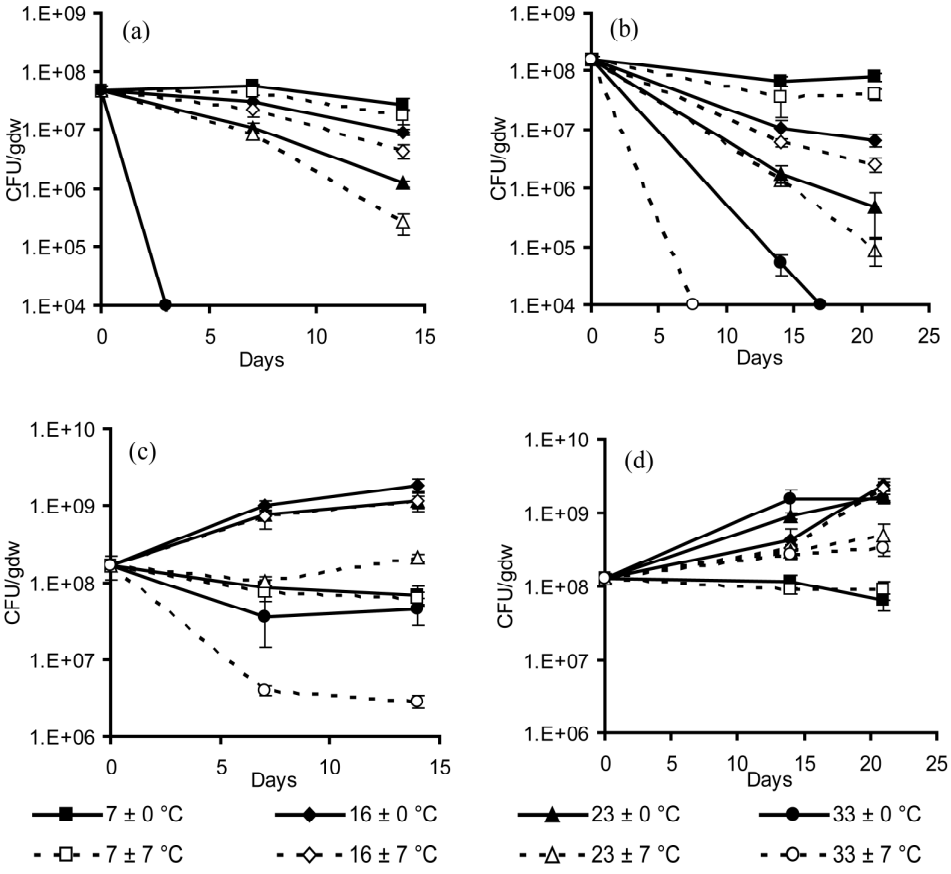

Fig. 1. Survival of *E. coli* O157:H7 (a, c) and *Salmonella* serovar Typhimurium (b, d) at different temperature levels for treatments with static temperatures and with ± 7 ºC oscillations in untreated (a, b) and sterilized manure (c, d). Data for treatments with ± 4 ºC oscillations are not shown. Vertical bars represent standard deviations.

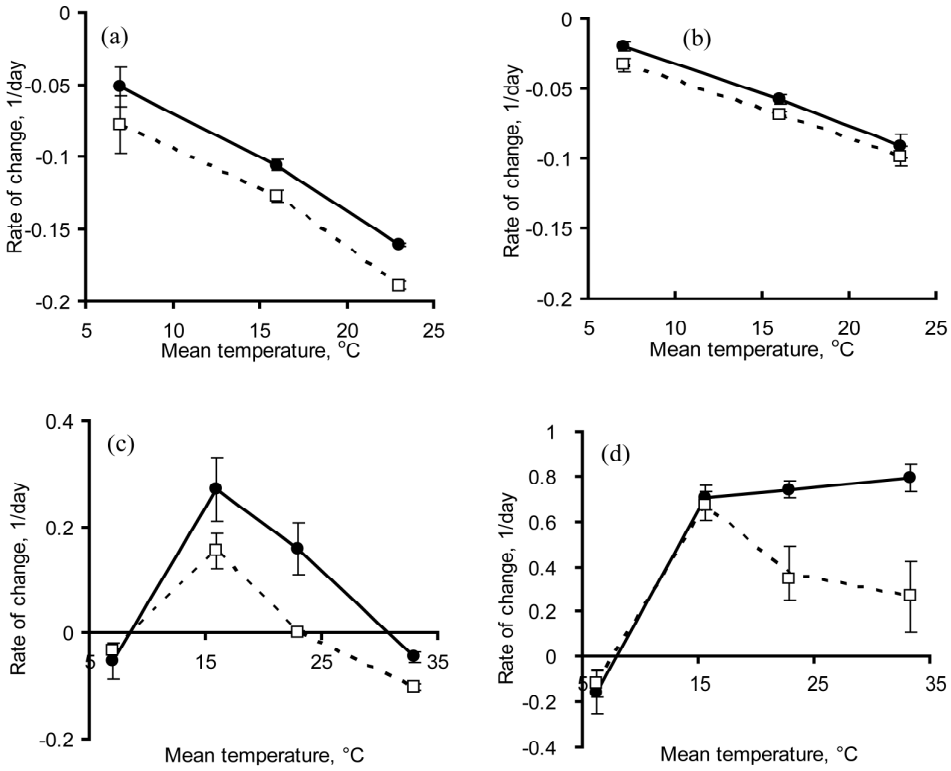

Fig. 2. Relative rates of change (day^{-1}) for *E. coli* O157:H7 (a, c) and *Salmonella* serovar Typhimurium (b, d) at three or four mean temperatures (7, 16, 23 and 33 ºC) with oscillation (± 7 ºC) (-□-) and without oscillation (–●–) in untreated (a, b) and sterilized manure (c, d). Vertical bars represent standard deviations.

effects of temperature oscillations on survival of *E. coli* O157:H7 were significant ($p<0.05$).

When log CFUs in untreated manure were regressed over time by nonlinear logistic regression, there were significant fits ($p<0.01$) for all temperature treatments with an average pseudo-r^2 of 0.89 ± 0.03. The estimated rates of change (parameter *m*) were normally distributed (Shapiro-Wilk test: $p=0.12$). Linear regression analysis showed that increasing mean temperature (MT) and its amplitude (AT) resulted in a significantly more negative rate of change (*m)* according to the following equation: *m* (model $r^2 = 0.96$; $p<0.001$) = $– 6.92 \times 10^{-3} \times$ MT ($p<0.001$) $– 3.54 \times 10^{-3} \times$ AT ($p<0.05$) (Fig. 2a). Differences among temperature treatments in calculated rates of change (*m*) of *E. coli* O157:H7 populations in sterilized manure (significant fits $p<0.01$

with an average pseudo-r^2 of 0.85 ± 0.15) were not significant by linear regression. However, t-tests for each mean temperature, except 7 ºC, showed significant ($p<0.05$) differences between treatments with and without temperature oscillations (Fig. 2c). The rates of change were smaller with than without oscillations, meaning that growth was slower at oscillating temperatures than at static temperatures (16 and 23 ºC), while the declines in population (7 and 33 ^{o}C) were faster in sterilized manure (Fig. 2c).

Influence of static and oscillating temperatures on CFUs of *Salmonella* serovar Typhimurium in manure. The greatest changes in CFUs of *Salmonella* serovar Typhimurium per g dry weight of manure (gdw^{-1}) occurred between the day of inoculation and the second week (Fig. 1b). The relations between the survival of *Salmonella* serovar Typhimurium and temperature were similar to those observed for *E. coli* O157:H7. Two weeks after addition of *Salmonella* serovar Typhimurium to non-sterilized manure (at 8.19 ± 0.06 log CFU gdw^{-1}), the density of this enteropathogen had decreased slightly to 7.82 ± 0.08 log CFU gdw^{-1} at a constant temperature of 7 ºC. Temperature oscillations led to a more rapid decline down to 7.69 ± 0.14 and 7.48 ± 0.26 log CFU gdw^{-1} at 7 ± 4 and 7 ± 7 ºC, respectively, although only CFU density of the last temperature treatment was significantly different from the other treatments with an average temperature of 7 ºC ($p<0.05$). At a constant temperature of 16 ºC, survival was significantly less than at 7 ºC, down to 7.00 ± 0.19 log CFU gdw^{-1}. Under oscillating temperatures survival was even less, down to 6.80 ± 0.18 and 6.77 ± 0.07 log CFU gdw^{-1} at 16 ± 4 and 16 ± 7 ºC, respectively. CFUs decreased to 6.23 ± 0.16 log CFU gdw^{-1} within 2 weeks at 23 ºC. In treatments with oscillating temperatures the changes were more pronounced, resulting in densities of 6.10 ± 0.38 and 6.07 ± 0.25 log CFU gdw^{-1} at 23 ± 4 and 23 ± 7 ºC, respectively. In contrast to *E. coli* O157:H7, *Salmonella* serovar Typhimurium was detected by plate counting after two weeks at 33 ºC at a density of 4.64 ± 0.42 log CFU gdw^{-1}, but only in the static variant. At all mean temperatures there were significant differences between fixed and oscillating temperatures, especially with amplitudes of ± 7 ºC.

In sterilized manure samples, there was a significant increase in CFUs at all temperature treatments after two weeks, except at 7 °C (Fig. 1d). The densities at 7 °C were 7.96 ± 0.06 and 8.07 ± 0.09 log CFU gdw^{-1} after two weeks of oscillating and constant temperatures, respectively, compared to the initial density of 8.10 ± 0.70 log CFU gdw^{-1}. At 16 and 16 ± 7 ºC, populations increased to 8.59 ± 0.17 and 8.51 ± 0.03 log CFU gdw^{-1}. Similar population densities were obtained after incubation for two weeks at 23 and 23 ± 7 ºC, namely 8.95 ± 0.04 and 8.47 ± 0.08 log CFU gdw^{-1}, respectively. Finally, in contrast to *E. coli* O157:H7, *Salmonella* serovar Typhimurium showed the greatest increase at 33 and 33 ± 7 ºC to a density of 9.16 ± 0.17 and 8.40 ± 0.15 log CFU gdw^{-1}, respectively, after two weeks.

Nonlinear logistic regression of population densities over time resulted in significant fits ($p<0.01$) with an average pseudo-r^2 of 0.85 ± 0.13. The normally distributed rates of change (Shapiro-Wilk test: $p=0.71$) were regressed on mean temperatures and their amplitudes by GLM analysis. The regression model for rate of change had significant mean temperature and amplitude effects: m (model $r^2 = 0.98$; $p<0.001$) = 1.42×10^{-2} ($p<0.001$) – $4.56 \times 10^{-3} \times$ MT ($p<0.001$) – $2.31 \times 10^{-3} \times$ AT ($p<0.01$) (Fig. 2b). Similar to effects of constant and oscillating temperatures on *E. coli* O157:H7, positive rates of change (significant fits $p<0.01$ with an average pseudo-r^2 of 0.84 ± 0.14) for *Salmonella* serovar Typhimurium were higher in sterilized manure under static than oscillating temperatures, but only in the case of mean temperatures of 23 and 33 ºC (Fig. 2d).

Influence of static and oscillating temperatures on direct counts of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in non-sterilized manure. At constant temperatures, the cell densities of *E. coli* O157:H7 counted with the epi-fluorescent microscope decreased slightly from 7.75 ± 0.20 log cells gdw^{-1} to 7.64 ± 0.14 log cells gdw^{-1} at 7 ºC, to 7.30 ± 0.19 log cells gdw^{-1} at 16 ºC, and to 7.29 ± 0.24 log cells gdw^{-1} at 23 ºC after two weeks. In two of nine manure samples stored at 33 ºC, one or two *E. coli* O157:H7 cells were found, but the numbers were too low to give meaningful averages. The ANOVA test with log-transformed populations (or survival ratios) indicated that there were significant influences of mean temperature and time of sampling ($p<0.05$), but there were no significant effects of temperature oscillations, probably because of low sensitivity of this method for populations close to the detection threshold.

Densities of *Salmonella* serovar Typhimurium cells decreased faster at increasing temperatures. As in all previous experiments, initial densities of 7.53 ± 0.06 log cells gdw^{-1} did not change significantly at 7 ºC, but changed to 7.34 ± 0.16 log cells gdw^{-1} at 16 ºC, to 7.18 ± 0.11 log cells gdw^{-1} at 23 ºC, and to 6.97 ± 0.13 log cells gdw^{-1} at 33 ºC. Mean temperature as well as time of sampling had significant effects ($p<0.05$) on the density of *Salmonella* serovar Typhimurium cells, but effects of temperature amplitudes were not significant.

Influence of static and oscillating temperatures on densities of total cultivable bacteria in non-sterilized manure. Densities of total bacterial CFUs on LB plates without antibiotics were similar after one, two and three weeks of incubation at all different treatments of oscillating and static temperatures, averaging 8.56 ± 0.08 log CFU gdw^{-1}. No significant influence of temperature, its oscillations and time of sampling on density of total bacteria was found. There was also no significant change from the initial density of 8.45 ± 0.10 log CFU gdw^{-1}. Initially, the added enteropathogens made up a significant proportion of the total bacteria, in some cases

more than 10%. After 2 weeks, *Salmonella* serovar Typhimurium or *E. coli* O157:H7 concentrations were on average less than 1% of the densities of total bacterial CFUs.

Estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. The survival period in manure ranged between less than 7 days for *E. coli* O157:H7 at 33 ºC to 159 days (theoretical estimation, viz. the number of days needed to reach 1 log CFU gdw^{-1}) at 7 ºC (Table 2). For *Salmonella* serovar Typhimurium, the survival period, predicted from the model, ranged from 227 days at 7 ºC to less than 21 days at 33 ºC (Table 3). Oscillating temperatures decreased survival more than expected from the same mean temperatures under static temperature conditions, especially at higher mean temperatures. After two weeks incubation of inoculated untreated manure at oscillating temperatures (±7 ºC), the populations of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were on average 52.6 and 39.0% reduced compared to those at static temperatures. In sterilized and inoculated manure incubated for 2 weeks, populations of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were on average 56.2 and 48.8% reduced at oscillating compared to constant temperatures. These percentages are about three times higher than daily variation in populations of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium under constant temperature conditions (A.V. Semenov, unpublished data). Effects of oscillations were not significant for cell densities counted under the epi-fluorescent microscope, but the sensitivity of this method was much lower than that of dilution plating.

Discussion

Similar to other studies on survival of enteropathogens in manure (Wang *et al.*, 1996; Kudva *et al.*, 1998; Himathongkham *et al.*, 1999; Wang *et al.*, 2004), survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in untreated manure decreased at increasing temperatures. Survival of *E. coli* O157:H7 was 1.44 ± 0.17 times shorter than that of *Salmonella* serovar Typhimurium under all temperature conditions, and the temperature effect was stronger for *E. coli* O157:H7 than for *Salmonella* serovar Typhimurium (Tables 2 and 3). A similar difference in survival between *Salmonella* serovar Typhimurium and *E. coli* O157:H7 had been shown previously for various substrates such as soil, slurry and manure (Himathongkham *et al.*, 1999; Franz *et al.*, 2005). However, for the first time, we showed that the temperature effect was stronger for *E. coli* O157:H7 than for *Salmonella* serovar Typhimurium in a natural substrate, viz. manure.

Although there are only scant data about the influence of temperature fluctuations on growth and survival of bacterial populations, it is well known that oscillating temperatures with the same mean as static temperatures influence growth and development of cold-blooded organisms differently compared to constant

temperatures. For example, the development time of *Drosophila melanogaster* increased, and body weight as well as growth rate decreased, under fluctuating (at 23 ± 5 °C with period of 1 day) compared to constant temperatures (Economos and Lints, 1986). The same effects were found for insect cells systems (Chang *et al.*, 1998). This is comparable to our results, namely reduced survival at oscillating temperatures. The development of a fungal plant pathogen under oscillating temperatures was simulated by theoretical modelling (Scherm and van Bruggen, 1994). The development was delayed under oscillating compared to constant temperatures, the delays being more pronounced around the temperature optimum than at minimal temperatures. The effects of mean temperatures were reduced as the temperature amplitudes increased (Scherm and van Bruggen, 1994). Survival of *Fusarium oxysporum* and *Sclerotium rolfsii* was also significantly different under fluctuating temperature and relative humidity compare to constant conditions (Shlevin *et al.*, 2003). In our experiments with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, greater differences in relative populations were also obtained between oscillating and constant temperatures, especially at higher than at lower mean temperatures (Fig. 1). In addition, the reduction in survival of either pathogen was more pronounced at amplitude of 7 ºC than of 4 ºC.

Contrary to the declining populations in untreated manure, populations of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium initially increased at lower temperatures in sterilized manure, indicating that the microbial community has an overriding effect on survival of these enteropathogens. *E. coli* O157:H7 seemed to be more sensitive to competition by the native microbial community than *Salmonella* serovar Typhimurium, as the temperature effects in untreated manure relative to those in sterilized manure were stronger for *E. coli* O157:H7. The optimum temperature for growth/survival of both pathogens was lower in untreated than in sterilized manure. Apparently, at intermediate (16-23 °C) and high (33 °C) temperatures, microorganisms antagonistic to enteropathogens are more competitive than at low temperatures, possibly because of faster growth or increased production of antimicrobials at higher temperatures. As the total bacterial populations were independent of incubation temperature, there must have been shifts in microbial composition with the changes in temperature (Panswad *et al.*, 2003). Although both pathogens were able to grow (as opposed to survive) in sterilized manure, growth was reduced compared to sterile broth, especially at temperatures near the optimum (37ºC) (A.V. Semenov, unpublished data). This suggests that availability of low-molecular weight substrate may have been more limiting in manure than in nutrient broth, so that there was strong competition for nutrients among cells of the same species in manure, especially at higher temperatures.

Dynamic temperature changes likely have a large influence on the rate of chemical reactions and the autochthonous microbial community as well as on introduced pathogen populations in cow manure. Yet, the mechanism of the greater effects of varying temperatures on survival and adaptation of the pathogens to a new

environment compared to static temperatures is still unclear. We hypothesize two possible explanations for the greater reduction in survival under oscillating than under constant temperatures: a mathematical and a physiological explanation. First, the non-linearity of the temperature response implies a relatively greater sensitivity to temperatures temporarily higher than the mean compared to temperatures temporarily lower than the mean (Scherm and van Bruggen, 1994). Second, an increase in temperature may constitute a greater stress and energy expenditure for a particular microorganism than a decreasing temperature. This would also hold for many autochthonous microorganisms, but the debilitating effects of oscillating temperatures may not hold for the antagonistic microbial community, as its composition likely shifts with the changes in temperature.

In natural eco-systems, such as fields and composting heaps, temperature is never static. Our results showed that survival of enteropathogens in manure under fluctuating temperatures is different compared to survival under static temperatures. Therefore, the predicted survival time and risk assessment can be overestimated if based on static temperatures. The nonlinear regression models for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium developed in this study could be used for a risk assessment model to predict survival of the pathogens in farm-yard manure under dynamic temperatures. Although in natural conditions the density of enteropathogens is usually around 10^4 - 10^5 CFU gdw^{-1}, in some cases the density in contaminated fresh manure can reach 10^7 CFU gdw^{-1} (Fukushima and Seki, 2004). Our initial concentration of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was 10^7 CFU gdw^{-1}, representing the worst case scenario. The presence of the *gfp* plasmid does not affect the intrinsic characteristics of the strain, no significant behavior differences were observed between *gfp*-transformed strains and parent strains (Fratamico *et al.*, 1997; Römling *et al.*, 2000), and therefore the use of bacteria with *gfp*-expressing plasmid is appropriate. However, validation of the regression model presented here under field conditions with wild types of enteropathogen strains and naturally oscillating temperatures would be needed to enhance the accuracy of risk assessment.

In conclusion, it may be difficult to accurately predict growth and survival of these food borne pathogens if only effects of static temperatures are used in risk assessment models. The difference between a monotonous decline in pathogen populations in untreated manure and initial growth in sterilized manure illustrates the importance of the autochthonous microbial community for the decline in natural substrates. *E. coli* O157:H7 seemed more sensitive to microbial competition than *Salmonella* serovar Typhimurium, and was affected more by increasing and oscillating temperatures. The results obtained in this research will contribute to the development of a risk assessment model for the contamination of lettuce plants produced in manure amended soil (Franz *et al.*, 2005).

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Chapter 3

Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farmyard manure and slurry

Semenov, A.V., van Overbeek, L., Hidayah N., Termorshuizen, A.J., and van Bruggen, A.H.C. (2008) Influence of aerobic and anaerobic conditions on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in Luria-Bertani broth, farmyard manure and slurry.
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Abstract

The influence of aerobic and anaerobic conditions on the survival of the enteropathogens *Escherichia coli* O157:H7 and *Salmonella* serovar Typhimurium was investigated in small microcosms with broth, cattle manure or slurry. These substrates were inoculated with a green fluorescent protein transformed strain of the enteropathogens at 10^7 cells g^{-1} dry weight. Survival data was fitted to the Weibull model. The survival curves in aerobic conditions generally showed a concave curvature, while the curvature was convex in anaerobic conditions. The estimated survival times showed that *E. coli* O157:H7 survived significantly longer under anaerobic than under aerobic conditions and ranged from approx. 2 weeks for aerobic manure and slurry to more than six months for anaerobic manure. On average, in 56.3% of the samplings (90% for aerobically and 40.1% for anaerobically stored manure and slurry), the number of recovered *E. coli* O157:H7 cells by anaerobic incubation of Petri plates was significantly ($p < 0.05$) higher in comparison with aerobic incubation. Survival of *Salmonella* serovar Typhimurium was not different between aerobic and anaerobic storage of LB broth or manure as well as between aerobic and anaerobic incubation of Petri dishes. The importance of changes in chemical composition and autochthonous microbial communities was also shown for the survival of *E. coli* O157:H7 in different oxygen conditions.

Introduction

The spread of enteropathogenic bacteria in the environment and food chains has increased in recent years (Flint et al., 2005; Rangel et al., 2005). It has been estimated that food-borne diseases cause approx. 76 million illnesses, 325,000 hospitalizations and 5000 deaths each year in United States (Mead et al., 2000). Many of the outbreaks have been linked to raw vegetables and fruits (Beuchat, 2002). Enteropathogens such as *Escherichia coli* O157:H7 and *Salmonella* serovar Typhimurium have been associated with the consumption of these products (Hilborn et al., 1999; Sivapalasingam et al., 2004). *E. coli* O157:H7 can be a serious treat for human health due to its low infective dose and severe clinical symptoms (Tilden Jr et al., 1996), while *Salmonella* serovar Typhimurium is less pathogenic but widespread in the world. Both pathogens are of great public concern (Beuchat, 1996; Joseph et al., 2002).

Cattle are known to be a major reservoir for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (Fossler et al., 2005; Hussein and Sakuma, 2005). Therefore, reducing survival of these pathogens in cattle manure is an important task for decreasing the risk of contamination of plant products and thus reducing the risk of food-borne diseases (Franz et al., 2005). Possible contamination of vegetables will largely depend on the survival capabilities of the pathogens in cattle faeces, in soil and on plants.

The survival and spread of *E. coli* O157:H7 is greatly affected by the way manure is stored and applied (Nicholson et al., 2005). Various characteristics of farm-yard manure and slurry, such as chemical composition (Franz et al., 2005; Franz et al., 2007; Franz et al., 2008), microbial community composition (Jiang et al., 2002; Semenov et al., 2007), and environmental factors during storage like pH (Himathongkham et al., 1999) and temperature (Wang et al., 1996; Wang et al., 2004; Fremaux et al., 2007; Semenov et al., 2007) lead to variation in the survival of *E. coli* O157:H7. Generally, *E. coli* O157:H7 survives longer at low temperature, acidic pH and high levels of dissolved organic carbon (DOC) (Franz et al., 2005). The presence or absence of oxygen in manure or slurry may also lead to differences in survival of the pathogen. *E. coli* O157:H7 survived for more than 1 year in a non-aerated ovine manure, while in similar aerated manure survival time was limited to 4 months (Kudva et al., 1998). In another study, *E. coli* O157:H7 was detected during 42 days in turned bovine manure heaps and at least for 90 days in unturned manure (Fremaux et al., 2007). In both studies, manure was incubated under fluctuating environmental conditions with continuous drying of the manure top layers, which may also affect survival. *Salmonella* serovar Typhimurium is known to be a more resistant to different environmental stresses and generally survives for longer periods of time in natural substrates than *E. coli* O157:H7 (Semenov et al., 2007).

The microbial community also has a great influence on the survival of enteropathogens in manure and slurry. *E. coli* O157:H7 survived longer in autoclaved soils amended with manure than in nonautoclaved soils (Jiang et al., 2002), indicating that competition must play an important role. The same differences in survival were also shown for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in farm-yard manure (Semenov et al., 2007). Significant shifts in the autochthonous microbial community in manure after storage were recently shown (Leung and Topp, 2001; Snell-Castro et al., 2005). We thus hypothize that in aerobic and anaerobic conditions the microbial community would be different and exert a different influence on survival of enteropathogens.

Since foods are routinely checked for the presence of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, several detection methods have been developed to screen for both pathogens. Selective plating is the most commonly applied procedure to screen for both pathogens in food. Several selective media have been described for their detection (De Boer, 1998). The most widely accepted method for quantitative screening of *E. coli* O157:H7 involves usage of sorbitol MacConkey (SMAC) agar (March and Ratnam, 1986). Selectivity for *E. coli* O157:H7 can be improved by addition of specific components such as cefixime and potassium tellurite (Zadik et al., 1993). For *Salmonella* serovar Typhimurium LB agar has been used, since the optimal medium has not yet been found (De Boer, 1998). In general, sensitivity is assumed to be very high, even though it was shown that higher numbers of pathogens (especially *E. coli* O157:H7) could be enumerated by direct cell counting methods as compared to plating methods, presumably due to viable but not culturable condition of some cells under standard plating conditions (Semenov et al., 2007). Incubation of Petri dishes in aerobic conditions at 37-42 ºC is the standard, while the digestive tract of animals, the natural reservoir of enteropathogens, is anaerobic and *E. coli* O157:H7 as well as *Salmonella* serovar Typhimurium are known as facultative anaerobic bacteria (Minor, 1984; Orskov, 1984). At the present time there is insufficient information about the direct influence of oxygen levels on the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium as well as the indirect influence through changes in the autochthonous microbial community and chemical reactions in manure and slurry. It is also not known whether common aerobic incubation of Petri dishes with the pathogens may cause underestimation in the number of cells recovered on selective media. We hypothesize that mimicking the environmental conditions in the digestive track of cows would result in higher cell recovery.

The objectives of our study were: 1) to assess the possible influence of anaerobic and aerobic conditions on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium; 2) to examine the survival of *E. coli* O157:H7 during storage of cow farm-yard manure and slurry in small microcosms under aerobic and anaerobic conditions; 2) to compare two isolation methods with Petri dishes incubated aerobically

and anaerobically. We also monitored for changes in physico-chemical (pH, moisture content, DOC, DON etc.) as well as in microbial diversity (Shannon diversity, *H*) and bacterial species richness (*S*) characteristics of farm-yard manure and slurry.

Materials and Methods

Bacteria. *E. coli* O157:H7 strain B6-914 *gfp*-91 was provided by Pina M. Fratamico (Fratamico et al., 1997). The strain had been modified from strain SEA 13B88 (from the outbreak linked to Odwalla apple cider; Food and Drug Administration), so that it contained genes coding for the production of green fluorescent protein (*gfp*) (pGFP cDNA vector) and ampicillin resistance, while genes coding for Shiga-like toxins ($Stx1^-$ and $Stx2^-$) were deleted. These changes did not result in any significant differences in survival in nutrient media compared to the wild-type strain (Fratamico et al., 1997; Kudva et al., 1998). *Salmonella* serovar Typhimurium MAE 119 (ΔagfD101 *saw*) was obtained from Römling (Römling et al., 1998; Römling et al., 2000). This strain carried resistance to kanamycin and gentamycin and carried the *gfp* gene after transformation with plasmid pAG408 mini-transposon. No differences between the wild-type of *Salmonella* serovar Typhimurium and its transformed form were found (Römling et al., 1998). Green fluorescence of the *gfp*-transformed strains were checked under UV light. Stock cultures of both strains were stored in 30% (w/w) glycerol at -80°C.

Manure and slurry. Farm-yard manure and slurry were collected from organically managed Friesian Holstein steers on a standard 50% grass/clover-silage + 50% dried grass diet at an organic farm at Bennekom, The Netherlands. Fresh manure without urine was mixed with straw (90% manure and 10% straw (kg/kg, dry weight) and stored (30-40 °C at 20 cm depth) for nearly one month in a heap. About 3 kg of manure from this heap as well as 3 litres of slurry from the farm reservoir were collected in February 2007, homogenized and stored in closed plastic bags at 5 °C for two weeks before the start of the experiment.

Inoculation of manure and slurry. Bacterial inocula were grown in Erlenmeyer flasks containing 150 ml fresh two-times diluted LB broth, with 50 μg ml^{-1} ampicillin (Sigma-Aldrich Chemie Gmbh, Germany) for *E. coli* O157:H7 and with kanamycin (50 μg/ml) for *Salmonella* serovar Typhimurium, followed by incubation at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The number of cells ml^{-1} of suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm in 1 ml cuvet was equal to 1×10^9 CFU ml^{-1}. Prepared inocula were added with a pipette to manure or slurry and mixed thoroughly within a double layer of plastic autoclavable bags. After thorough kneading of plastic bags by hand for 5 min., 50 g of

the inoculated manure or slurry was moved to Erlenmeyer flasks with a final density of 10^7 CFU per gram of manure/slurry dry weight (gdw^{-1}). To prevent drying, all flasks were closed with cotton wool.

Setup of experiments. Three types of experiments were conducted to investigate the sensitivity of aerobic and anaerobic plating procedure as well as the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in aerobic and anaerobic conditions. The first set of experiments (Exp. 1) were carried out in sterilized two-times diluted LB broth stored aerobically and anaerobically for two weeks. Petri plates were incubated aerobically and anaerobically. Inoculated LB broth was incubated at 16 ºC and sampling was done at time zero and after 2, 6, 9 and 12 days. A supplementary experiment was conducted in two-times diluted LB broth amended with 50 µl of manure extract, to mimic the influence of microbial competition on survival of *E. coli* O157:H7. This manure extract was prepared by filtering a 1 : 5 (g/v) manure suspension through a 0.8µm filter to eliminate protozoa.

The second set of experiments was carried out to investigate if recovery of *Salmonella* serovar Typhimurium and *E. coli* O157:H7 from a natural substrate like manure incubated for 2 and 5 days is different on agar plates in aerobic versus anaerobic conditions (Exp. 2).

The third set of experiments was carried out with *E. coli* O157:H7 only, because there was no effect of aerobic versus anaerobic conditions on survival of *Salmonella* serovar Typhimurium in LB broth (Exp. 1) nor in farm yard manure (Exp. 2), while there were significant effects of storage conditions on survival of *E. coli* O157:H7 in those substrates. In the last set of experiments (Exp. 3) survival of *E. coli* O157:H7 was compared for manure and slurry stored in aerobic and anaerobic conditions. In addition, CFUs of *E. coli* O157:H7 on agar plates incubated under aerobic and anaerobic conditions were compared. Four treatments (manure and slurry inoculated with *E. coli* O157:H7 stored in aerobic/anaerobic conditions) were incubated for 60 days at 16 ºC. Each treatment had three replicates. Two subsamples per treatment were dilution plated two times per week during the first month and weekly during the second month (see under sampling procedure). For each subsample, half of the plates were incubated aerobically, half anaerobically.

Anaerobic conditions. To create anaerobic conditions, oxygen was chemically bound by Anaerocult A (Merck KGaA, Germany) inside of special jars (GasPak System, Merck KGaA, Germany). The absence of oxygen was checked by the Anaerotest (Merck KGaA, Germany). The blue oxidized form of the dye methylene blue is converted in oxygen-free (anaerobic) medium into the (colourless) leucomethylene blue. In the presence of oxygen the reduced leucobase passes again into the oxidized from (blue) One anaerobic jar contained one Erlenmeyer flask with inoculated sample

(3 reps). For anaerobic incubation of Petri plates, 12 of them were placed inside of an anaerobic jar and stored at 37 ºC.

Sampling procedure. For the experiments with LB broth (Exp. 1), 0.5 ml of broth was added to 4.5 ml of sterile distilled water for preparation of a ten-fold dilution series. In experiments with natural substrates (Exp. 2 and 3), samples of manure or slurry, approximately 0.5 g fresh weight, were put in pre-weighed dilution tubes with 4.5 ml of sterile distilled water to determine the exact weight. Samples were vortexed and sonicated for 30 s (Branson 5200, 120-W output power, 47 kHz). Ten-fold dilution series were prepared with sterile distilled water, and 50 µl of the two highest dilutions per sample was plated in duplicate on sorbitol-MacConkey agar (Oxoid) with ampicillin (50 µg ml^{-1}) for *E. coli* O157:H7 and on LB agar with kanamycin (50 µg/ml) for *Salmonella* serovar Typhimurium, respectively. After adding approximately 20 sterile glass beads per Petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass beads to spread the inoculum over the surface of the plate. Petri dishes were incubated aerobically for 24 h and anaerobically for 72 h at 37 °C till all colonies are developed to be counted. Fluorescent bacterial colonies were counted under a UV lamp (365 nm UV-A, PL-S, Philips, Eindhoven, the Netherlands) after incubation. Fluorescent colonies made up 95-99% of the total number of colonies on a plate. Fluorescent colony-forming units (CFU) were calculated per ml of broth (for Exp. 1) or per gram of dry manure/slurry (for Exp. 2 and 3). The calculated detection limit was 2.2 and 2.8 log CFU gdw^{-1} for plating of manure and slurry samples, respectively.

When the density of *E. coli* O157:H7 was lower than the detection limit in Exp. 3, the enrichment procedure was used to check if there was any survival at all. For enrichment, 20 g of manure or slurry was added to 180 ml of modified EC broth (Fluka Chemie GmbH, Switzerland). Enrichments were incubated at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Subsamples of 50 µl were plated as described above.

Finally, densities of total cultivable bacteria were also determined in Exp. 3 by preparing dilution series as described above. Subsamples of 50 µl were plated in duplicate on LB medium and incubated aerobically for 24 h at 37 ºC. All colonies with typical bacterial morphology were counted.

Chemical characterization. Fresh uninoculated samples of manure and slurry from the control pots without enteropathogens were analyzed at the start and at the end of experiment 3. The pH was measured in water suspension (1 : 2.5 g/v) with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3^-), ammonium (NH_4^+) and total dissolved nitrogen (N_{ts}) content were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic nitrogen (DON) was calculated as the

difference between N_{ts} and the amount of nitrogen present as NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Dried (24 h at 40 °C) manure and slurry samples were ground and used to measure total carbon (C_{total}) and total nitrogen (N_{total}) by the Dumas method followed by detection by a Fisons element analyzer type EA 1108 (Therom Finnigan Italia S.P.A., Milan, Italy).

Denaturing Gradient Gel Electrophoresis. DGGE analyses were conducted in order to relate the survival of *E. coli* O157:H7 in manure and slurry to shifts in bacterial community composition. DNA was extracted from 300 mg (fresh weight) manure or slurry with the Bio101® Systems FastDNA® SPIN® Kit according to the manufacturer's specifications (Qbiogene, Inc., Carlsbad, CA, USA). The 16S rRNA genes of eubacteria were amplified from manure/slurry DNA with the eubacterial primer pair U968-GC and L1401 (Felske et al., 1996). Eubacterial PCR was performed using a touchdown scheme for 30 thermal cycles and followed by a final extension step at 72 °C for 30 min (Janse et al., 2004). The PCR products were analysed in standard 1.2 % (w/v) agarose-0.5x Tris-borate-EDTA (TBE) gel stained with ethidium bromide staining, to confirm presence and appropriate size of the PCR-amplified products. DGGE was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). For the gradient 6% acrylamide gels (37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient (Muyzer et al., 1993) were used to separate the generated amplicons (100% denaturant is 7 M urea and 40% formamide). Electrophoresis was performed in 0.5x TAE buffer for 16 h at 100 V at a constant temperature of 60 °C. Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The gels were scanned using ScanSoft Omnipage pro. 14 at a resolution of 300 dots per inch. Scanned gels were analysed with TotalLab TL120 (version 2006a, NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). Bands were selected manually. Data of different DGGE gels were standardized by referring to the DGGE marker. The 16S rDNA fragments detected by DGGE were considered to represent the most numerous bacterial groups, making up at least 0.1-1% of the total community (Muyzer et al., 1993). The bacterial diversity was estimated as species richness, *S*; as well as the Shannon-Wiener index of bacterial diversity, *H*. Species richness *S* was defined as the number of DGGE detected bands per soil type. The Shannon-Wiener diversity index was calculated as $H = -\sum P_i \log P_i$ based on the relative band intensities as formulated by Eichner *et al*. (Eichner et al., 1999). DGGE analysis was done in duplicate where the replicas were on different gels. *S* and *H* were calculated as the mean of the two replicas.

Statistical analysis. The number of colonies for each Petri plate was expressed in CFU g dw^{-1} and standard deviations were calculated for every treatment. To describe the

decline in CFUs over time (for Exp. 1 and 3), log-transformed data were fitted (separately for each replication) to the Weibull survival model (Gauss-Newton method): $\log (N / N_0) = - (t / b)^n$, where $\log (N / N_0)$ is the relative population size (CFU gdw^{-1}) at time t (days), b (scale parameter) represents the *time of first decimal reduction* (days) and n is a shape parameter (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). For $n>1$ a convex curve is obtained, while for $n<1$ a concave curve is obtained. This model is based on the assumption that the cells resistance to stress, as encountered in manure or slurry, follow a Weibull distribution and that the survival curve is the cumulative form of this underlying distribution of individual inactivation kinetics (van Boekel, 2002). Model performance was assessed by calculating the regression coefficient (R^2) and significance. In addition to the model parameters, the survival time needed to reach the detection limit (2.2 and 2.8 log CFU gdw^{-1} for farm-yard manure and slurry, respectively) was calculated (*survival time* in days). The two-sided t-test was used to distinguish differences in survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium between aerobic and anaerobic conditions as well as to determine the influence of aerobic/anaerobic incubation of sampled Petri dishes. MANOVA analysis was done to determine possible interrelation between aerobic and anaerobic storage of inoculated substrate (LB broth (Exp. 1), farm-yard manure and slurry (Exp. 2 and 3)) and aerobic and anaerobic incubation of Petri dishes. The nonparametric χ^2 – test was used for a comparison of cases when *E. coli* O157:H7 and *Salmonella* serovar Typhimurium CFU density was significantly higher for anaerobically than for aerobically incubated Petri plates.

Results

Model performance. All curves of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium survival were characterised by different shape, curvature and rate of decline (Fig 1A-C, 2A-B). Therefore a flexible model had to be chosen for satisfactory analysis. Only the Weibull survival model showed a mean R^2 of 0.97 ± 0.01 for all treatments (farm-yard manure, slurry and LB broth) and experiments (Exp. 1 and 3). No significant differences in R^2 among the treatments were found. Observed and modelled values of the CFU of enteropathogens were positively correlated ($r = 0.97$, $p < 0.001$), indicating that the Weibull model can fit survival curves in all cases well. All other tested survival models (exponential, logistic etc) had unacceptable variation in R^2 for some of the treatments.

Influence of oxygen on survival of *Salmonella* serovar Typhimurium in LB broth and manure and on colony growth on Petri plates. Initial inoculation density of *Salmonella* serovar Typhimurium was 9.23 ± 0.11 log CFU ml^{-1} for sterilised LB broth (Exp. 1). The pathogen population declined significantly during 12 days of storage (Fig 1C). Decline of *Salmonella* serovar Typhimurium was not significantly different

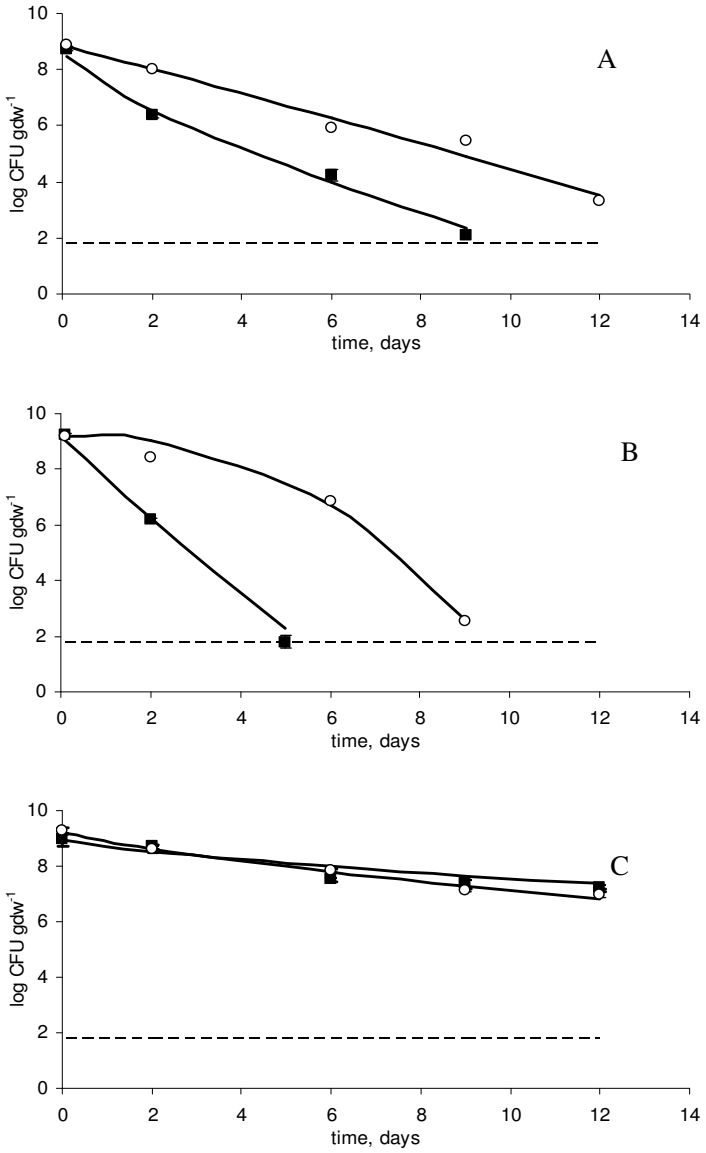

Fig. 1. Representative example of fitting a Weibull survival model (solid line) to observed survival data for *E. coli* O157:H7 (A, B) (Exp. 1) and *Salmonella* serovar Typhimurium (C) (Exp. 1) under aerobic (closed squares) and anaerobic (open circles) conditions for sterilized LB broth (A, C) and sterilized LB broth with manure extract (B). Standard errors are not shown since vertical bars are too small to be visible on the graph.

($p > 0.05$) in aerobic and anaerobic storage conditions with an average reduction of 2.8 log CFU ml^{-1} during 12 days.

Anaerobic incubation of Petri plates did not have a significant effect on *Salmonella* serovar Typhimurium compared with aerobic incubation. For aerobically stored LB broth cultures, anaerobic incubation of Petri dishes resulted in significantly higher counts only in one case of sampling, while for anaerobically stored LB broth cultures no significant differences were found between incubation treatments of Petri dishes. When log CFUs of *Salmonella* serovar Typhimurium were regressed over time using the Weibull survival model, there were significant fits ($p < 0.05$) for all treatments and replicates with an average pseudo-R^2 0.95 ± 0.02. Estimated *survival time* of *Salmonella* serovar Typhimurium was similar ($p > 0.05$) during anaerobic storage (74.1 ± 32.1 days) and aerobic storage (68.2 ± 25.5 days). The survival curves in sterilised LB broth showed convex curvature.

In the experiment with farm-yard manure (Exp. 2), manure was inoculated with *Salmonella* serovar Typhimurium at 8.2 log CFU g dw^{-1}. Samplings after 2 and 5 days of incubation did not show significant differences between aerobic and anaerobic storage of manure (average decline after 5 days was 1.7 log CFU g dw^{-1}) ($p = 0.67$) as well as between aerobic and anaerobic incubation of Petri dishes ($p = 0.75$).

Influence of oxygen on survival of *E. coli* O157:H7 in LB broth and on colony growth on Petri dishes. Immediately after inoculation, the density of *E. coli* O157:H7 was 8.91 ± 0.10 log CFU ml^{-1} for sterilised LB broth (Exp. 1). Pathogen populations declined significantly during the 12 days of storage (Fig 1A). In aerobic storage condition, *E. coli* O157:H7 reached its detection limit after 9 days, while in anaerobic condition decline was significantly ($p < 0.05$) less with 6.4 log CFU ml^{-1} reduction during 12 days.

In LB broth with microbial competitors from farm-yard manure, decline of the *E. coli* O157:H7 population was significantly faster in comparison with decline in sterilised LB broth (Fig. 1B). Initial density of *E. coli* O157:H7 was 9.18 ± 0.07 log CFU ml^{-1}. *E. coli* O157:H7 could not be detected by plate counting already after 5 days and 9 days for aerobically and anaerobically stored samples, respectively.

Anaerobic incubation of Petri plates resulted in significantly higher *E. coli* O157:H7 CFU/ml compared with the standard procedure of aerobic incubation. For aerobically stored LB broth cultures anaerobic incubation on Petri plates resulted in more counts in 80% of all cases, while for anaerobically stored LB broth cultures, the number of such cases was two times lower, only 40%. For cultures in LB broth with manure extracts stored in aerobic conditions, 50% of the samplings had significantly higher numbers of *E. coli* O157:H7 CFUs when Petri plates were incubated anaerobically, while 75% of such cases were registered for anaerobically stored LB broth cultures (Table 1).

When log CFUs for Exp. 1 were regressed over time using the Weibull survival model, there were significant fits ($p < 0.01$) for all treatments and replicates with an average pseudo-R^2 0.98 ± 0.01. *Survival time* of *E. coli* O157:H7 was significantly higher during anaerobic storage compared to aerobic storage, both for sterilized LB broth and LB broth with manure extracts (Table 3). Only in case of anaerobic storage of sterilised LB broth, *survival time* was significantly different ($p < 0.05$) between aerobic and anaerobic incubation of Petri plates (15.2 ± 0.2 and 21.3 ± 0.5 days, respectively). While the survival curves in sterilised LB broth showed concave curvature, for LB broth with manure extracts convex curvature was more prevalent.

Table 1. Number of samplings when density of *E. coli* O157:H7 CFU's on plates incubated in anaerobic condition was significantly higher / not different / significantly lower than those on plates incubated in aerobic condition, after storage of inoculated LB broth, manure or slurry in aerobic or anaerobic conditions (Exp. 1 and 3).

Substrate	Storage method of inoculated samples	
	aerobic	anaerobic
LB broth	4 / 1 / 0	2 / 3 / 0
LB broth [1]	1 / 1 / 0	3 / 1 / 0
Manure	4 / 0 / 0	4 / 7 / 0
Slurry	5 / 1 / 0	5 / 6 / 0

[1] 50 µl of manure extract was added to provide microbial competition for *E. coli* O157:H7 (Exp. 1)

Table 2. Estimated survival time (days) for *E. coli* O157:H7 needed to reach the detection limit in LB broth, manure and slurry in aerobic and anaerobic conditions (Exp. 1 and 3)

Estimated survival time ± SE, days				
Substrate	Storage method of inoculated samples			
	aerobic		anaerobic	
	Incubation method of Petri plates		Incubation method of Petri plates	
	aerobic	anaerobic	aerobic	anaerobic
LB broth	9.6 ± 0.4^{a}	10.9 ± 0.1^{a}	15.2 ± 0.2^{b}	21.3 ± 0.5^{c}
LB broth [1]	5.2 ± 0.1^{a}	5.3 ± 0.1^{a}	9.4 ± 0.2^{b}	9.5 ± 0.1^{b}
Manure	12.0 ± 1.3^{a}	13.0 ± 1.4^{a}	183.8 ± 18.9^{b}	177.7 ± 3.21^{b}
Slurry	15.7 ± 1.0^{a}	18.7 ± 1.2^{b}	45.2 ± 1.2^{c}	45.7 ± 0.8^{c}

[1] 50 µl of manure extract was added to provide microbial competition for *E. coli* O157:H7 (Exp. 1)

[a, b, c] significant ($p < 0.05$) differences among storage and incubation treatments within each substrate

Influence of oxygen on survival of *E. coli* O157:H7 in farm-yard manure and slurry and on colony growth on Petri dishes. In experiment 2, farm-yard manure was inoculated with *E. coli* O157:H7 at 7.9 log CFU g dw^{-1}. After 2 and 5 days of anaerobic incubation of manure, there were significant differences between aerobic and anaerobic incubation of Petri dishes ($p < 0.05$).

Density of *E. coli* O157:H7 in farmyard manure (Exp. 3) decreased from 8.03 ± 0.12 log CFU g dw^{-1} at the start of the experiment to 5.85 ± 0.06 log CFU g dw^{-1} after 47 days of anaerobic storage. *E. coli* O157:H7 stored in aerobic manure was detectable on the 12^{th} day only on anaerobically incubated Petri plates. However, the qualitative standard aerobic enrichment procedure showed the presence of *E. coli* O157:H7 during the next 21 days (Fig. 2A).

Generally, the same behavior of *E. coli* O157:H7 was observed in slurry as in manure (Exp. 3). Two months after addition of *E. coli* O157:H7 to this substrate (at 7.93 ± 0.03 log CFU g dw^{-1}), the density of the pathogen decreased to 3.37 ± 0.06 log CFU g dw^{-1} after anaerobic storage. Aerobic storage led to a significantly more rapid decline. *E. coli* O157:H7 could be detected up to 15 days by aerobic plating, up to 19 days on Petri plates incubated anaerobically and for the next 7 days by the enrichment procedure (Fig. 2B).

The influence of anaerobic incubation of Petri plates in case of manure and slurry was similar to that observed for LB broth. On average, for 56.3% of the samplings (90% for aerobically and 40.1% for anaerobically stored manure and slurry) the density of *E. coli* O157:H7 was significantly ($p < 0.05$) higher when Petri dishes were incubated in anaerobic conditions in comparison with common aerobic plating (Table 1).

Nonlinear regression of population densities with the Weibull survival model resulted in significant fits ($p < 0.001$) with an average pseudo-R^2 of 0.98 ± 0.02. The survival period in manure and slurry ranged from 12 to 183 days, depending on the oxygen conditions. In anaerobic slurry, estimated *survival time* was significantly higher ($p < 0.01$) than in aerobic slurry (on average, 45.5 and 17.2 days, respectively). In farm-yard manure the difference in *E. coli* O157:H7 survival was more than 14 times ($p < 0.001$): 181 days in anaerobic manure and 12.5 days in aerobic manure (Table 3). No interaction was found between manure storage conditions and incubation conditions of Petri dishes.

Differences in manure/slurry characteristics after aerobic and anaerobic storage. At the end of experiment 3 the pH and NO_3^- concentrations were significantly higher for aerobically stored substrates ($p < 0.05$) while organic matter content, NH_4^+ and dissolved organic carbon concentrations were higher ($p < 0.05$) in anaerobic manure and slurry (Table 3).

The presence of oxygen in manure significantly decreased the diversity and

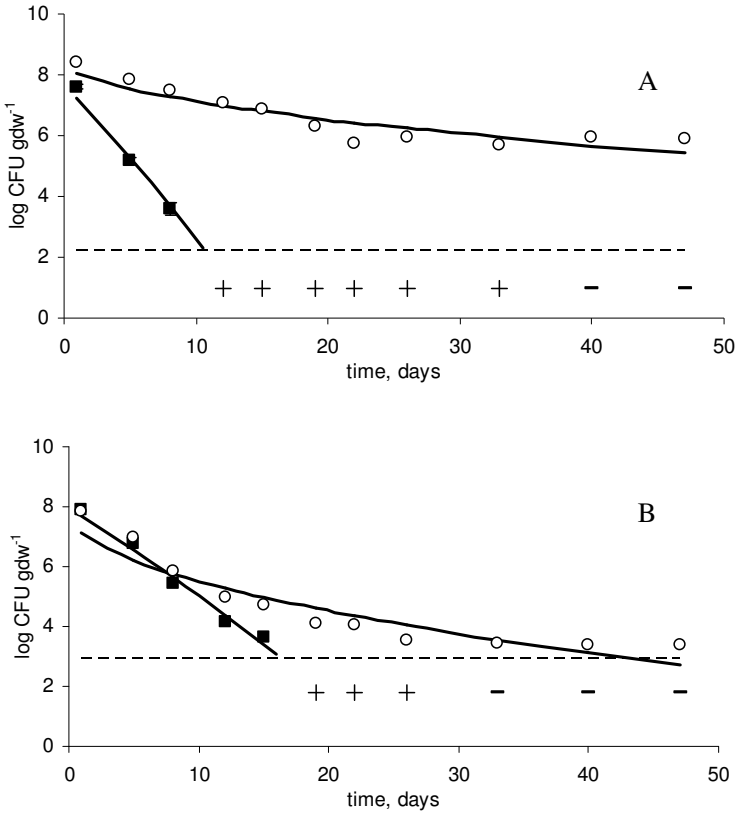

Fig. 2. Representative example of fitting a Weibull survival model (solid line) to observed survival data for *E. coli* O157:H7 under aerobic (closed squares) and anaerobic (open circles) conditions for farm-yard manure (A) and slurry (B) (Exp. 3). "+" represent positive samples after enrichment, while "-" represent negative samples. Standard errors are not shown since vertical bars are too small to be visible on the graph.

species richness of the bacterial community in comparison with anaerobically stored manure (Table 1). Contrary to that, *S* and *H* indexes increased ($p < 0.05$) in aerobic and anaerobic slurry treatments at the end of the experiment. Total bacteria were lower ($p < 0.05$) at the end of the experiment in comparison with initial numbers (for aerobic and anaerobic storage) with average decreases of 2.02 ± 0.37 and 0.59 ± 0.05 log CFU gdw^{-1} for manure and slurry, respectively (Table 3).

DGGE profile comparisons of PCR-amplified 16S DNA showed that although differences in microbial community composition due to storage time were relatively

Table 3. Chemical and biological characteristics of manure and slurry at the beginning and at the end of the experiment (Exp. 3)

Variable	Substrate					
	Farm-yard manure			Slurry		
	-[1]	aerobic[2]	anaerobic[2]	-[1]	aerobic[2]	anaerobic[2]
Physico-chemical data						
Dry matter content, %	35.20[a]	36.10[a]	35.80[a]	5.50[a]	6.00[b]	5.80[b]
pH	9.10[a]	9.50[b]	6.20[c]	8.50[a]	9.30[b]	8.20[a]
Organic matter, g kg^{-1}	74.75[a]	66.78[b]	72.49[a]	60.93[a]	56.87[b]	53.10[c]
N-NH_4, g kg^{-1}	1.00[a]	0.16[b]	1.04[a]	0.60[a]	0.14[b]	0.56[a]
N-NO_3, g kg^{-1}	0.00[a]	0.03[b]	0.00[a]	0.00[a]	0.73[b]	0.00[a]
DOC, g kg^{-1}	5.02[a]	4.39[b]	4.74[a]	12.48[a]	6.95[b]	10.56[c]
DON, g kg^{-1}	0.17[a]	0.19[a]	0.17[a]	0.37[a]	0.56[b]	0.26[a]
Biological data						
Shannon index, *H*	1.31[a]	1.00[b]	1.37[a]	1.21[a]	1.32[b]	1.30[b]
Species richness, *S*	24.50[a]	11.80[b]	30.30[a]	20.50[a]	28.30[b]	28.50[b]
Total bacteria, log CFU gdw^{-1}	9.23[a]	6.82[b]	7.59[c]	8.45[a]	7.81[b]	7.91[b]

[1] at the beginning of the experiment.

[2] at the end of the experiment after 2 month of aerobic/anaerobic storage.

[a, b, c] significant ($p < 0.05$) differences among storage treatments within each substrate.

large, overall the greatest differences were caused by aerobic or anaerobic storage for farm-yard manure or slurry (Fig. 3).

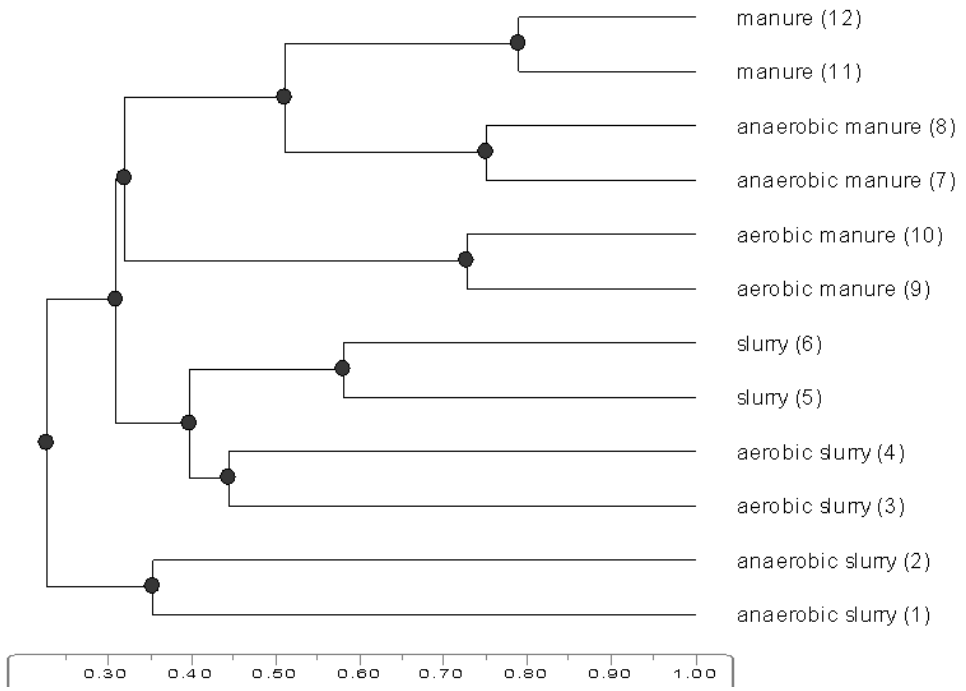

Fig. 3. Dendrogram of DGGE patterns of bacterial DNA extracted from manure and slurry (Exp. 3) incubated in aerobic and anaerobic conditions for 2 months (generated by TotalLab TL120). Samples of slurry (5, 6) and manure (11, 12) were collected at the beginning of the experiment. The similarity of patterns was calculated by Pearson correlation with weighting of band intensities. The scale bar indicates levels of similarity. Duplicate samples were used.

Discussion

Similar to other studies on survival of *E. coli* O157:H7 in farm-yard manure and slurry (Kudva et al., 1998; Nicholson et al., 2005; Fremaux et al., 2007), the survival was longer in nonaerated than in aerated substrates, while for *Salmonella* serovar Typhimurium this difference was not significant (Nicholson et al., 2005). For the first time, we showed that the presence of oxygen can have a direct effect on survival of *E. coli* O157:H7 as well as an indirect effect through changes in chemical processes and in the autochthonous microbial community composition. The influence of anaerobic storage on survival of *E. coli* O157:H7 was more pronounced in farm-yard

manure than in slurry. This seems logical since under aerobic conditions oxygen concentrations in the more liquid incubated slurry are likely to be lower than in farm-yard manure. In addition, anaerobic incubation of Petri dishes with sorbitol-SMAC agar resulted in significantly higher numbers of recovered *E. coli* O157:H7 cells in comparison with the standard procedure. *Salmonella* serovar Typhimurium did not show a significant difference in survival for aerobically and anaerobically stored substrates as well as for aerobic and anaerobic incubation of Petri dishes.

The survival time of *E. coli* O157:H7 (Exp. 1 and 3) and *Salmonella* serovar Typhimurium (Exp. 1) was estimated by the Weibull model. This model proved to be suitable for describing the decline of *E. coli* O157:H7 in soils amended with manure (Franz et al., 2008) and for the survival of soil borne plant pathogens (Shlevin et al., 2003). In the present study, decline of *E. coli* O157:H7 had concave ($n > 1$) and convex ($n < 1$) shapes in aerobic and anaerobic conditions, respectively. *Salmonella* serovar Typhimurium had a convex shape in both conditions. Populations with a convex shape of the survival curve tended to be more adaptive to initial environmental changes since the decrease in population over time was less pronounced.

E. coli O157:H7 survived anaerobic conditions in manure or slurry for significantly longer periods in comparison with aerobic conditions. Therefore, anaerobically stored manure and slurry should not be distributed on fields where there is a possibility of contamination of plants with human pathogens (Kudva et al., 1998). Aeration resulted in accelerated drying of manure and this was believed to be responsible for the reduction in pathogen density (Kudva et al., 1998). In our experiments, moisture content was at the same level during the whole experimental time; therefore drying could not have been responsible for the shorter survival of *E. coli* O157:H7 in aerobically stored manure.

To investigate if oxygen level and not moisture content had an effect on growth and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, two experiments with LB broth were carried out. In both cases, *E. coli* O157:H7 showed significantly longer survival time in anaerobic conditions compared with aerobic conditions. Presence of natural competitors, extracted from farm-yard manure, decreased the survival time of *E. coli* O157:H7 approximately by a factor 2, but did not influence the shape of survival curves. Moreover, in 62% of sampling cases, anaerobically incubated Petri dishes had significantly higher numbers of *E. coli* O157:H7 colonies. *Salmonella* serovar Typhimurium did not have such differences in survival and recovery level. This corroborates earlier research showing that *Salmonella* serovar Typhimurium is more resistant to various environmental stresses (Franz et al., 2005; Semenov et al., 2007). Thus, aeration can affect survival of *E. coli* O157:H7 regardless of drying effects, while this is generally not the case for *Salmonella* serovar Typhimurium.

Absence or presence of oxygen has a large influence on intensity and directions of chemical reactions (Masse and Droste, 1997; Veeken et al., 2002), on the autochthonous microbial community as well as on introduced enteropathogen populations in farm-yard manure or slurry (Tiquia et al., 2002). Since *E. coli* O157:H7 is a facultative anaerobic organism, it is generally assumed that this pathogen can survive better in anaerobic conditions (Kudva et al., 1998; Nicholson et al., 2005; Fremaux et al., 2007). Based on our results, we hypothesize that there is a complex influence of oxygen condition on pathogen survival through a combination of interactions between chemical processes and changes in the microbial community.

In previous studies it was shown that a decrease in pH (Franz et al., 2005) as well as an increase in DOC (Franz et al., 2008) had a positive effect on survival of *E. coli* O157:H7. We showed that the presence or absence of oxygen can play a major role in changes in pH and DOC over time. Fermentation processes which occur only in anaerobic conditions lead to the production of organic acids followed by a decrease in pH (Kemmitt et al., 2006). Fast utilization of DOC is possible only in the presence of easily available nitrogen sources (such as NO_3^- and NH_4^+) and oxygen. However, in anaerobic conditions denitrification takes place resulting in N_2O and N_2. Although hydrolysis of compounds containing organic nitrogen can occur both in aerobic (by ammonification and nitrification) and in anaerobic conditions (by heterotrophic nitrification and denitrification), absence of nitrates in anaerobic condition may indicate that denitrification may use all available nitrates (Sørensen, 2001). Contrary to this, in aerobic conditions mineralisation takes place with the production of ammonium, which increases the pH (Kemmitt et al., 2006). Aerobic mineralisation is more effective than anaerobic, therefore the decrease in DOC is more pronounced (Bengtsson et al., 2003; Burger and Jackson, 2003). pH and DOC as well as other environmental characteristics apparently do not have pronounced effects on survival of *Salmonella* serovar Typhimurium and changes in chemical reactions may not have a similar influence on survival of *Salmonella* serovar Typhimurium as was found for *E. coli* O157:H7.

Profound biochemical changes are associated with significant shifts in microbial community composition as detected by DGGE in manure and slurry (Exp. 3). The community composition for manure at the beginning of the experiment and after storage in anaerobic conditions was relatively similar. This can be explained by conservation of conditions in manure similar to those in the gastrointestinal tract. Similarly, 33% of pig slurry phylotypes were closely related to phylotypes retrieved from the pig gastrointestinal tract (Snell-Castro et al., 2005). However, aeration promoted a 150-fold increase in aerobic phylotypes, while most of the phylotypes originally present in the manure declined in relative intensity during aerated incubation (Leung and Topp, 2001). Aerobic mineralization of carbon sources may lead to higher numbers of copiotrophic bacteria and therefore a less complex structure of the

microbial community, as found after aerobic storage of farmyard manure (Table 3). In case of slurry, the structure of the microbial community at the beginning of the experiment was more similar to the structure in slurry after 2 months of aerobic storage than to that after anaerobic storage. Since pronounced mineralization in aerobic conditions takes place only in the top part of slurry the differences between aerobically and anaerobically stored slurry were not as extreme as for farm-yard manure. Possible immobilization of nutrients by copiotrophic bacteria in aerobic condition intensifies the competition between the autochthonous microbial community and *E. coli* O157:H7, while relatively high levels of easily available sources in anaerobic condition may increase the survival of *E. coli* O157:H7 in manure and slurry.

Selective media for quantification of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium have been compared to increase the sensitivity of planting procedures (De Boer, 1998; Vidovic et al., 2007). However, in all cases Petri dishes were incubated under aerobic conditions, while *E. coli* O157:H7 and *Salmonella* serovar Typhimurium are facultative anaerobic bacteria. We propose that transfer of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium cells from mostly anaerobic conditions (in manure and slurry) to aerobic conditions (during plating procedures and incubation of Petri dishes) may lead to oxygen stress and lower the recovery rate of enteropathogens. In our experiment, 56.3% of the samplings of *E. coli* O157:H7 (90% for aerobically and 40.1% for anaerobically stored manure and slurry) resulted in significantly ($p < 0.05$) higher numbers of recovered cells during anaerobic incubation of Petri plates in comparison with the common aerobic incubation. This was not the case for *Salmonella* serovar Typhimurium: the number of recovered cells was the same after anaerobic and aerobic incubation of Petri dishes in the LB broth experiment (Exp. 1) as well as in the farm-yard manure (Exp. 2). To prevent an underestimation of *E. coli* O157:H7 numbers during surveys and experiments used for risk assessments, Petri dishes should be incubated anaerobically, while for *Salmonella* serovar Typhimurium the common aerobic procedure could be still appropriate.

In conclusion, the difference in decline of *E. coli* O157:H7 between anaerobically and aerobically stored manure and slurry illustrates the important role of autochthonous microbial community and chemical processes for the survival of this pathogen. Moreover, the difference in response of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium to a transition from anaerobic to aerobic conditions indicates that *E. coli* O157:H7 has a lower physiological adaptability to oxygen availability than *Salmonella* serovar Typhimurium. In areas where *E. coli* occurs a well-aerated soil might be a management tool that contributes to minimize potential hazards.

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Chapter 4

Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils

Franz, E., Semenov, A.V., Termorshuizen, A.J., de Vos, O.J., Bokhorst, J.G., and van Bruggen, A.H.C. (2008) Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils.
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Abstract

The recent increase in foodborne disease associated with the consumption of fresh vegetables stresses the importance of the development of intervention strategies that minimize the risk of preharvest contamination. To identify risk factors for *E. coli* O157:H7 persistence in soil we studied the survival of a Shiga-toxin defficient mutant in a set of 36 Dutch arable manure-amended soils (organic/conventional, sand/loam) and measured an array of biotic and abiotic manure-amended soil characteristics. The Weibull model, which is the cumulative form of the underlying distribution of individual inactivation kinetics, proved to be a suitable model for describing the decline of *E. coli* O157:H7. The survival curves generally showed a concave curvature, indicating changes in biological stress over time. The calculated time to reach the detection limit *ttd* ranged from 54 to 105 days and the variability followed a logistic distribution. Due to large variation among soils of each management type, no differences were observed between organic and conventional soils. Although the initial decline was faster in sandy soils, no significant differences were observed in *ttd* between both sandy and loamy soils. With sandy, loamy and conventional soils the variation in *ttd* was best explained by the level of dissolved organic carbon per unit biomass carbon *DOC/biomC*, with prolonged survival at increasing *DOC/biomC*. With organic soils the variation in *ttd* was best explained by the level of dissolved organic nitrogen (positive relation) and the microbial species diversity as determined by DGGE (negative relation). Survival increased with a field history of low-quality manure (artificial fertilizer and slurry) compared to high-quality manure application (farmyard manure and compost). We conclude that *E. coli* O157:H7 populations decline faster under more oligotrophic soil conditions, which can be achieved by the use of organic fertilizer with a relatively high C/N ratio and consequently a relatively low rate of nutrient release.

Introduction

During the last three decades, an increasing number of outbreaks caused by foodborne pathogens has been associated with the consumption of fresh produce (IFT/FDA, 2003; Sivapalasingam et al., 2004). Recently, a large multi-state outbreak of *E. coli* O157:H7 due to the consumption of contaminated fresh spinach in the USA resulted in 187 cases of illness (including 97 hospitalizations and three deaths) (Anonymous, 2006). Produce can become contaminated at any point during the primary production, processing and distribution. For pathogens with animal reservoirs, like VTEC *E. coli* O157:H7, contamination in the field can occur when contaminated manure is used for fertilization. *E. coli* O157:H7 may become associated with the surface of vegetables grown in contaminated manure-amended soil (Natvig et al., 2002; Islam et al., 2004) where they can survive and even grow (Abdul-Raouf et al., 1993). Recently it has been demonstrated that *E. coli* O157:H7 can become internalized in the plant during growth in contaminated soil (Solomon et al., 2002; Franz et al., 2007), which may constitute a public health risk since these bacteria are unlikely to be removed during post-harvest sanitation or washing by consumers.

Prevention of preharvest contamination of fresh produce is an essential part of a systems approach focused on applying interventions designed to achieve delivery of microbiologically safe produce to consumers (Beuchat, 2006). Suppression of human pathogens in manure-amended soil and the subsequent prevention of spread into the food chain by contamination of produce must be realized by intrinsic factors of the soil system since anti-bacterial pesticides applicable to soil are not available. In order to develop strategies that minimize the risk of pathogen survival and spread within the agricultural system and food chain, it is important to study the fate of *E. coli* O157 in environmental substrates like manure-amended soil and to understand how manure-amended soil conditions affect its survival (IFT/FDA, 2003).

Reported survival times of *E. coli* O157:H7 in manure-amended soil range between several weeks and more than 6 months, depending on soil type, bacterial strain and experimental setup (Jiang et al., 2002; Avery et al., 2004; Islam et al., 2004; Johannessen et al., 2005; Nicholson et al., 2005). Both abiotic (temperature, pH, soil moisture, soil type) and biotic (composition and diversity of the microbial community) factors affect the survival capabilities of bacteria introduced into the soil habitat (van Veen et al., 1997). Most studies considered the effects of soil characteristics independently. Since the extent to which these factors affect survival most likely depends on interactions between the various environmental factors, the overall set of abiotic and biotic soil characteristics should be taken into account. In order to assess which factors determine the fate of *E. coli* O157:H7 in manure-amended soil, its survival should be described quantitatively and as accurate as possible. The Weibull model is a flexible model to describe pathogen deactivation (van Boekel, 2002) and has previously been used to model the survival of soilborne plant pathogens under

solarisation (Shlevin et al., 2003) and the deactivation of *E. coli* O157:H7 in food products (Guan et al., 2006; Buzrul and Alpas, 2007; Chen, 2007). Multiple regression techniques can subsequently be used to identify those (a)biotic factors that explain most of the variation in the survival parameters, and to construct predictive models.

Only few attempts have been made to link survival of *E. coli* O157:H7 with soil physico-chemical and biological variables (Mubiru et al., 2000; Ogden et al., 2001; Jiang et al., 2002). Extrapolating results from studies conducted with commensal *E. coli*, which is often used as an indicator organism for faecal contamination, seems not entirely valid since these indicator organisms behave differently and may survive longer than *E. coli* O157:H7 (Fenlon et al., 2000; Mubiru et al., 2000; Durso et al., 2004). Moreover, the existence of naturalized soil-borne *E. coli* in densities up to 10^3 cfu/g confounds the use of this bacterium as a reliable indicator organism for pathogen spread and survival (Ishii et al., 2006).

Soil management can significantly affect soil characteristics and soil functioning (Bossio et al., 1998; van Diepeningen et al., 2006). While in conventional arable farming systems soil fertility is maintained with the use of synthetic fertilizers next to the use of organic amendments (e.g. manure), organic farmers refrain from the use of synthetic fertilizers and soil fertility is built up solely with organic amendments (frequently consisting of animal manure). Organically managed soils generally show higher microbial diversity, total of microbial activity and microbial biomass compared to conventional soils (Mäder et al., 2002; van Diepeningen et al., 2006). These differences have been associated with an enhanced suppression of soil-borne (fungal) plant pathogens (van Bruggen and Termorshuizen, 2003). Currently it is not known to which extent various soil management practices can influence the survival of human enteric pathogens, introduced by the amendment of soil with manure.

Recently, 12 pairs of neighboring organic and conventional agricultural soils in the Netherlands were compared with respect to biological and physico-chemical soil properties (van Diepeningen et al., 2006). In the present study 18 pairs of organic and conventional soils, including the above mentioned 12, were used to determine differences in decline kinetics of *E. coli* O157:H7 in a wide array of soils. More specifically, the goals were to (i) model the survival of *E. coli* O157:H7 in a range of soils with an appropriate inactivation model and quantify the variability in survival, (ii) determine the relative importance of management type and soil type with respect to the survival of *E. coli* O157:H7 and (iii) identify physical, chemical and/or biological manure-amended soil characteristics which are responsible for differences in decline kinetics.

Materials and Methods

Soil collection. Soil was collected from six SKAL (inspection body for organic production) accredited organic (coded ORG) farms with lettuce fields throughout the

Netherlands, representing to the best of our knowledge all lettuce producing organic farms in this country. Six conventional (coded CONV) farms with lettuce fields were selected as much as possible on comparable soil types, so that pairs of ORG and CONV farms could be formed. In addition, 12 SKAL accredited ORG farms with other crops were selected throughout the Netherlands (van Diepeningen et al., 2006). For each ORG farm a neighboring CONV farm was selected with an adjacent field, identical soil type and with the same crop (potato, grassland, sugar beet, wheat or maize). Each pair of ORG and CONV farms was sampled on the same day. Throughout each sampled field, 10 soil sub-samples (20 cm deep) were collected between the plants with an augur and mixed. Crop coverage at the moment of sampling was low on all 24 soils because of the low temperatures in early spring. Samples were collected during March, April and May 2005. All samples were transported to the laboratory in plastic bags, thoroughly mixed, sieved through 0.5 cm mesh to remove plant parts and earthworms and stored at 5 °C until the start of the experiment (May 2005).

Soil texture. Fractions of clay particles (<2 μm), silt particles (2-50 μm) and sand particles (50-2000 μm) were determined in all collected soils before mixing with manure by laser diffraction. Soils were classified into soil types according to a US texture diagram and summarized as being sand or loam.

Adjusting water content. To ensure similar water availability in the different soils the water content of each individual soil was adjusted to 60% of its maximum water holding capacity (WHC). The actual water content was measured by drying approximately 5 g soil for 24 h at 105 ^{o}C. The WHC of the soil was determined by adding an excess of distilled water to approximately 50 g of field-moist soil sample. The sample was left overnight covered with aluminium foil to prevent evaporation. The well-drained soil was filtered in a funnel with filter paper mounted on a collecting flask and again allowed to stand overnight covered with aluminium foil. Subsequently, the WHC was determined by drying the soil sample of approximately 5 g of well-drained soil for 24 h at 105 ^{o}C. The amount of water to be added was calculated by taking 60% of the WHC minus the water content of the field-moist sample. From each collected soil 1000 g was adjusted to a water content of 60% of the WHC. Subsequently, two portions of 450 g from each soil were placed in plastic bags. One portion was mixed with non-inoculated manure and used as a control on which all chemical and biological characteristics were determined (all at start of the experiment) and one portion was mixed with inoculated manure to study pathogen survival.

Chemical characterization. Dried (24 h at 40 ^{o}C) soil-manure samples from the control pots were ground, sieved through a 2 mm mesh and analyzed at the start of the

experiment. The pH was measured in water with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3), ammonium (NH_4) and total dissolved nitrogen (N_d) content were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic nitrogen (DON) was calculated as the difference between N_{ts} and the amount of nitrogen present as NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Total carbon (C_{total}) and total nitrogen (N_{total}) were measured by the Dumas method followed by detection by a Fisons element analyzer type EA 1108 (Therom Finnigan Italia S.P.A., Milan, Italy).

Copiotrophic and oligotrophic bacteria enumeration. Two samples from each control pot of approximately 1 g were suspended in 4.5 ml buffered peptone water (BPW), vortexed vigorously for 1 minute, sonicated in an ultrasonic cleaner (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT) for 30 s, vortexed again for 30 s and serially diluted in BPW. Fifty microliters of suitable dilutions were plated in duplicate on high and low carbon medium for quantification of respectively copiotrophic and oligotrophic bacteria (Semenov et al., 1999). The high carbon medium contained 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 1.3 g $K_2HPO_4 \cdot 3H_2O$, 0.06 g $Ca(NO_3)_2 \cdot 4H_2O$, 25 g glucose, 2 g enzymatic casein hydrolysate (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) and 17.0 g Agar no. 3 (Oxoid Limited, Basingstoke, UK) per liter. The low carbon medium was similar but with 1000 fold diluted carbon concentration. After incubation for 60 h on high-C medium (for copiotrophic bacteria) and 3 weeks on low-C medium (for oligotrophic bacteria), bacterial colonies were counted and colony-forming units (CFUs) were calculated per g dry soil.

Basal respiration. Microbial activity was assessed by measuring the basal respiration rate. Basal respiration of the manure-amended control soils was determined in duplicate with an automated system in which a continuous air-flow of 65 ml/min was led over 60-90 g of fresh weight soil in glass tubes (length 24 cm, diam. 3.5 cm) incubated at 20 ºC for 24 h. After passing over the soil, the moisture in the air was absorbed by special granulate and the CO_2-concentration was analyzed by means of a computer-controlled switching device and an infrared CO_2 analyzer (ADC 7000 analyser, Analytical Development Corporation, Hoddesdon, UK). Two empty tubes were used as controls to measure the concentration of CO_2 in the air. For calculation of the basal respiration the readings of the first 10 h of incubation were omitted. The respiration was expressed in µg CO_2/g dry weight/h.

Microbial biomass. Microbial carbon present in the manure-amended control soil was measured using the fumigation extraction method (Vance et al., 1987). The method is based on the assumption that the organic carbon measured after a 24 h $CHCl_3$-fumigation originates from the cells of the microbial biomass so that the difference between organic carbon extracted by 0.5 M K_2SO_4 from a fumigated and a non-fumigated sample of the same soil can be used to estimate soil microbial biomass. We used 2.22 instead of 2.64 as a factor to convert the carbon released by fumigation into biomass carbon since we used UV-persulphate as the analytical procedure to measure organic carbon instead of dichromate digestion (Wu et al., 1990). Microbial biomass was expressed as mg C / kg dry weight soil-manure mixture.

DGGE. DGGE analyses were conducted in order to relate the survival of *E. coli* O157:H7 to the species richness and species diversity of the microbial population present in the manure-amended soil. DNA was extracted from 300 mg (fresh weight) manure-amended soil with the Bio101® Systems FastDNA® SPIN® Kit for Soil according to the manufacturer's specifications (Qbiogene, Inc., Carlsbad, CA, USA) except that bead beating (three times 90 s) was used instead of the FastPrep1 instrument. The 16S rRNA gene of eubacteria were amplified from soil-manure DNA with the eubacterial primer pair U968-GC and L1401 (Felske et al., 1996). The eubacterial PCR was performed using a touchdown scheme for 30 thermal cycles and finished by an extension step at 72 °C for 30 min (Rosado et al., 1998; Janse et al., 2004). The PCR products were examined by standard 1.2 % (w/v) agarose-0.5x Tris-borate-EDTA (TBE) gel electrophoresis with ethidium bromide staining, to confirm product integrity and size. DGGE was performed using the DCode system (Bio-Rad Laboratories, Hercules, CA, USA). We used 6% acrylamide gels (37.5 acrylamide:1 bisacrylamide) with a 45–60% denaturing gradient (Muyzer et al., 1993) to separate the generated amplicons (100% denaturant is 7 M urea and 40% formamide) and an 8% acrylamide stack without denaturing agents. The gels were poured from the top in the DCode□ template, prepared with Gelbond PAG film (Amersham Pharmacia Biotech AG, Uppsala, Sweden) to one side, using a gradient maker and a Heidolph Pumpdrive (Heidolph, Schwabach, Germany) set at 4 ml/min. Eubacterial PCR products derived from DNA of each manure sample were loaded in adjacent slots.

Electrophoresis was performed in 0.5x TAE buffer for 16 h at 100 V at a constant temperature of 60 °C. Gels were stained with Bio-Rad's Silver Stain (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol, but using the protocol for gels >1 mm thick instead of 0.5–1 mm to compensate for the barrier formed by the Gelbond. After staining the gels were preserved for at least 1 h in Cairn's preservation solution of 25% ethanol (v/v) and 10% glycerol (v/v), covered by a permeable cellophane sheet (Amersham Pharmacia Biotech Ag, Uppsala, Sweden) and dried overnight at 60 °C.

The gels were scanned using ScanSoft Omnipage pro. 14 at a resolution of 300 dots per inch. Scanned gels were analysed with Phoretix 1D (NonLinear Dynamics Ltd., Newcastle upon Tyne, UK). Bands were selected manually. Data of different DGGE gels were standardized by referring to the DGGE marker. The 16S rDNA fragments detected by DGGE were considered to represent the most numerous bacterial groups, making up at least 0.1-1% of the total community (Muyzer et al., 1993). The bacterial diversity was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H*. Species richness *S* was defined as the number of DGGE detected bands per soil type. The Shannon-Wiener diversity index was calculated as $H' = -P_i \log P_i$ based on the relative band intensities as formulated by Eichner *et al*. (Eichner et al., 1999), where P_i (importance probability of the band) is defined as n_i/N where n_i is the area of the peak in intensity and N the sum of all peak areas in the lane profile. The Shannon-Wiener diversity index is a general diversity index which increases with the number of species and which is higher when the mass is distributed more evenly over the species. DGGE analysis was done in duplicate where the replicas were on different gels. *S* and *H* were calculated as the mean of the two replicas.

Strain. Strain *Escherichia coli* O157:H7 B6-914 GFP-91 was kindly provided by Dr. Pina Fratamico (Fratamico et al., 1997). This strain does not produce the Shiga-like toxins I or II ($Stx1^-$ $Stx2^-$), but contains the pGFP cDNA vector (Clontech Laboratories, Inc. Palo Alto, CA) expressing green fluorescent protein (GFP) and ampicillin resistance. The use of an GFP-expressing plasmid is legitimate since the presence of the plasmid does not affect the intrinsic characteristics of the strain, no significant behaviour differences were observed between GFP-transformed strains and parent strains and the stability/expression of the marker in unfavourable conditions was demonstrated (Fratamico et al., 1997; Tombolini et al., 1997; Vialette et al., 2004) . In addition, no difference in survival between toxin positive ($Stx1^+$ $Stx2^+$) and toxin negative ($Stx1^-$ $Stx2^-$) *E. coli* O157:H7 in bovine manure was observed (Kudva et al., 1998). Survival in manure of *E. coli* O157:H7 which passed the intestinal tract of cattle was not different from the survival of the same strain directly inoculated into the manure (Scott 2006). Bacteria were stored at –80°C and checked for viability prior to use by growing on Luria-Bertani medium supplemented with ampicillin (50 µg ml^{-1}).

Inoculation of manure and preparation of soil-manure mixtures. Manure from steers (MRIJ breed mixed with Montbéliarde breed) on a standard 50% grass/clover-silage + 50% dried grass diet was collected from a manure-straw heap (30-50^{o}C at 20 cm depth) at the organic experimental farm Droevendaal (Wageningen University and Research Centre, The Netherlands). Seventy-two portions of 50 g were weighted and put in small plastic bags.

Bacterial inocula were grown in Erlenmeyer flasks containing 150 ml buffered peptone water (BPW) supplemented with 50 µg/ml ampicillin, followed by incubation at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The number of cells per ml of suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm was equal to 1×10^9 CFU ml^{-1}. Cells suspended in BPW were added to half of the manure portions with a final density of 1×10^7 CFU per gram dry-weight of final soil-manure mixture, taking into account the water content of the manure and that of the individual soils. An identical volume of BPW was added to the control treatments. The manure and the inoculum were thoroughly mixed by kneading in a plastic bag from the outside by hand. Subsequently, the inoculated portions of manure were added to and thoroughly mixed with the 450 g portions of soil. The mixture was transferred to 1 L pots which were closed (but with the ability of gas exchange) and incubated at 16°C in darkness.

Sampling. The inoculated soil-manure mixtures were sampled nine times within 60 days after inoculation to determine the survival of the pathogens. At each sampling time two samples of approximately 1 g of each replica was removed from the middle of the mixture using a sterile spoon and put in separate pre-weighed dilution tube with 4.5 ml of 0.1% peptone. Sampling holes were closed. Sample-tubes were weighed to determine the exact size of the sample. Samples were vortexed and put in a Branson 5200 ultrasonic bath for 30 s. The samples were vortexed again and 10-fold serial dilutions were made. From the two highest dilutions 50 µl was plated in duplicate on Petri dishes with Sorbitol MacConkey (SMAC, Oxoid) agar supplemented with 50 µg/ml ampicillin (detection limit of 100 CFU/g dry weight). The number of necessary dilutions was estimated based on preliminary counts. Cell suspensions were spread on the surface by shaking with 2 mm sterile glass beads. The inoculated plates were incubated at 37 °C for 18 hours. Numbers of *E. coli* O157:H7 were determined after incubation for 18 hours at 37 °C by counting green fluorescent CFU's using a dark-blue lamp (Philips PL-S 9W/08 Blacklight Blue, peak at 365 nm UV-A). Colony counts were calculated to log number of CFU gdw^{-1}. The detection limit of the plating technique was approximately 100 CFU per gram dry weight of manure-amended soil.

Statistical analysis

Model description. Survival of *E. coli* O157:H7 was modelled by fitting the experimental data to the Weibull survival function (SAS® system for Windows version 8.02, SAS Institute Inc, Cary, NC, USA, 2001). This model is based on the assumption that the cells' resistances to stress, as encountered in the soil-manure mixture, follow a Weibull distribution and that the survival curve is the cumulative form of this

underlying distribution of individual inactivation kinetics (Mafart 2002, van Boekel 2002): $\log \frac{N}{N_0} = -\left(\frac{t}{b}\right)^n$

where log N/N_0 is the log number of the relative population size (CFU gdw^{-1}) at time *t* (days), *b* (scale parameter) represents the *time of first decimal reduction* (days) and *n* (shape parameter). For $n>1$ a convex curve is obtained, while for $n<1$ a concave curve is obtained. Model performance was assessed by calculating the regression coefficient (R^2) and the Root Mean Squared Error (RMSE). In addition, the residuals were subjected to a test for normality. The performance of the Weibull model was compared with a linear model. In addition to the model parameters, the time needed to reach the detection limit of 100 CFU gdw^{-1} was calculated (*ttd* in days).

Data analysis. Model parameters, physico-chemical and biological variables were checked for normality and transformed when necessary. Differences in physico-chemical and biological variables between ORG and CONV soils were assessed with paired t-tests. Differences between sand and loam and the four different combinations by independent sample t-tests. Pearson correlation matrices were constructed to reveal linear relations between model parameters and environmental variables. Multivariate analysis of variance (MANOVA) was conducted in order to reveal the effect of soil and management type on both Weibull model parameters simultaneously. Analyses were conducted in SPSS v 12 (SPSS Inc., Chicago, Illinois, USA). The set of obtained Weibull model parameters were tested for correspondence with theoretical distributions using the Anderson-Darling goodness of fit test (@Risk software version 4.5.4 Palisade Corporation). The variability in the model parameters were described by fitting the values to the highest ranking probability distribution. Multiple regressions were conducted with the statistical software package SAS with the 'stepwise' selection (significance level p=0.15). The following parameters were included in the analysis: percentage of sand particles (50-2000 µm) [*sand*], years being organically certified [*years*], microbial diversity as determined by DGGE [*H*], species richness as determined by DGGE [*S*], log (nitrate in mg/kg dw) [NO_3], log (ammonium in mg/kg dw) [NH_4], log (dissolved organic carbon in mg/kg dw) [*DOC*], log (dissolved organic nitrogen in mg/kg dw) [*DON*], log (dissolved organic carbon / dissolved organic nitrogen) [*DOC/DON*], log (C/N) [*C/N*], log (ratio number of copiotrophic bacteria over number of oligotrophic bacteria) [*copio/oligo*], log (biomass carbon in mg/kg dw) [*biomass*], log (moisture content in percentage of total weight) [*moist*], log (dissolved organic carbon per unit biomass carbon x 10) [*DOC/biomC*], log (dissolved organic nitrogen per unit biomass carbon x 10) [*DON/biomC*], and log (basal respiration per unit biomass carbon in mg/kg x 100) [CO_2*/biomC*]. The pH was included after the following normal transformation: arsin(sqrt(pH/pH_{max})) [*pH*]. In order to avoid

possible nonlinear relations between the dependent and independent variables because they do not share the same underlying distribution, regression analyses were conducted on normalized data. When variables included in the model were significantly correlated, the variable with the lowest contribution to the model was removed and the regression analysis was repeated to allow other variables to enter the model. Multiple regression was conducted for the whole set of 36 soils and for each management type and soil type separately. Alternative models were assessed by removing the main predictor and running the regression analysis again. Only those alternative models performing equally or better with respect to the amount of variation explained (R^2) were mentioned.

Results

Model performance. Although the survival curves were close to (log)linear, they showed a concave curvature (Fig. 1A). The Weibull model for survival in all 36 soils had a mean R^2 of 0.98 and a mean RMSE of 0.26 (log CFU) while the linear model showed a mean R^2 of 0.92 and a mean RMSE of 0.36. Residuals obtained from the Weibull model were normally distributed for all fits, displaying no systematic tendencies to be positive or negative. Although the residuals from the linear model were also normally distributed for all fits they deviated more from a normal distribution compared to the residuals of the Weibull model. Since the Weibull model performed best in describing the survival of *E. coli* O157:H7 in manure-amended soil and none of the fits were unsatisfactory, the Weibull model parameters were further analyzed. The observed and modelled values of *E. coli* O157:H7 density over time were highly correlated ($r=0.96$, $p<0.0001$) (Fig. 1B). The goodness-of-fit statistics (R^2 and RMSE) did not differ significantly between survival curves in sand compared to loam, ORG versus CONV and between the four combinations, indicating that the Weibull model is suitable to fit survival curves of *E. coli* O157:H7 in an array of different manure-amended soils equally well.

The variability in decline kinetics of *E. coli* O157:H7 in 36 different soils were assessed by histograms of the Weibull model parameters and by fitting the data to probability density functions (Fig. 2). Based on the Anderson-Darling (A-D) goodness-of-fit statistic, the variability in the Weibull parameters *b* and *n* could be best described by log-logistic distributions, while *ttd* could be best described by a logistic distribution (A-D test statistics respectively 0.325, 0.385 and 0.204; all $p>0.1$).

Effects of soil type and soil management on survival. The majority of the survival curves showed a concave shape, with a relatively fast initial decline followed by a slower decline phase (correlation between parameters *b* and *n* was 0.91, $p<0.001$). Survival times (determined as the time needed to reach the detection limit *ttd*) of *E. coli* O157:H7 was on average 79.8±12.7 days (S.E. of the mean = 2.11, 95%CI: 75.5 –

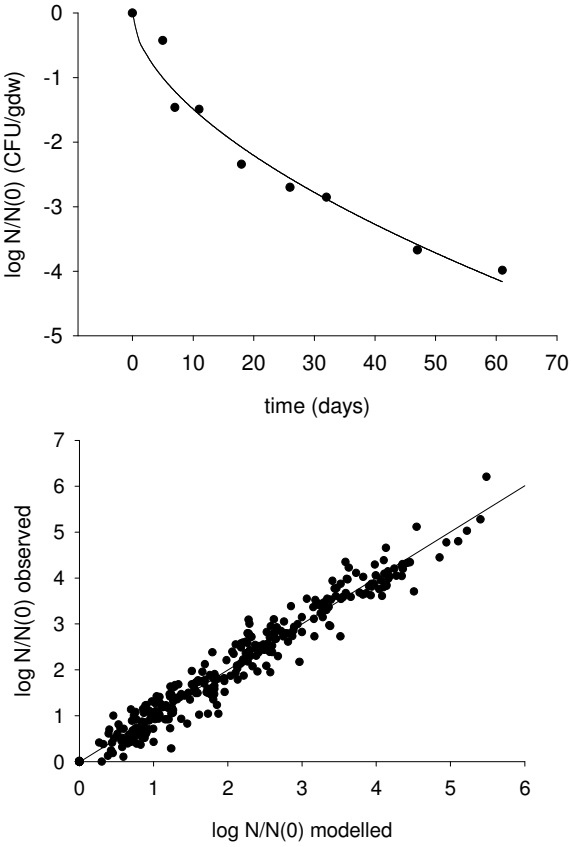

Fig. 1. A) representative example of *E. coli* O157:H7 survival in manure-amended soil showing observed values (circles) and fitted Weibull decline curve (solid line). B) plot of the correlation between observed and modelled values for log N/N_o for survival in all 36 soils.

84.1, lowest = 54, highest = 105) (Fig. 3A). Multivariate analysis of variance revealed that soil type affected the overall decline kinetics significantly ($p=0.006$, Wilks Lambda = 0.658). The first decimal reduction time *b* and the shape parameter *n* were higher for loamy soils (mean b = 10.3 days, mean n = 0.81) compared to sandy soils (mean b = 6.8 days, mean n = 0.64) (both $p<0.0001$) (Fig. 4). This combination of a faster initial decline and a more concave shape of the decline curve for sand compared to loam, resulted in crossing of the average survival curves of both soil types after 54 days (Fig. 3B). Although the overall survival, expressed as the time to detection level

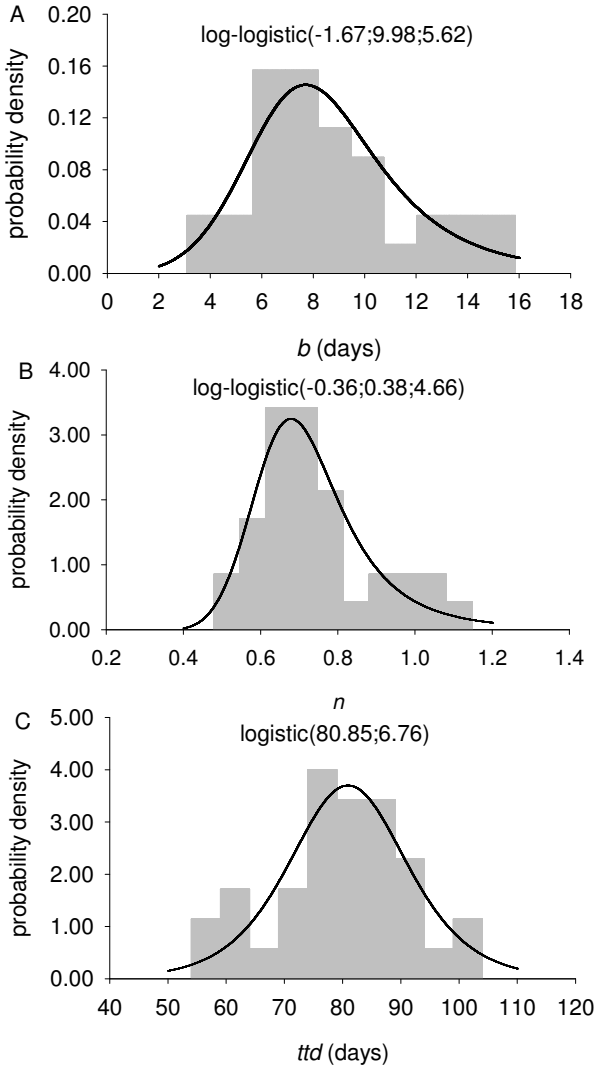

Fig. 2. Histograms and fitted probability distribution function of the Weibull decline curve parameters *b* (first decimal reduction time) (A), *n* (shape parameter) (B) and the derived *ttd* (time to detection limit) (C) for the survival of *E. coli* O157:H7 in 36 different soils.

ttd, was longer for sandy soils (mean = 84 days) compared to loamy soils (mean = 78 days), no significant difference in the *ttd* were observed (p=0.151) (Fig. 4). The

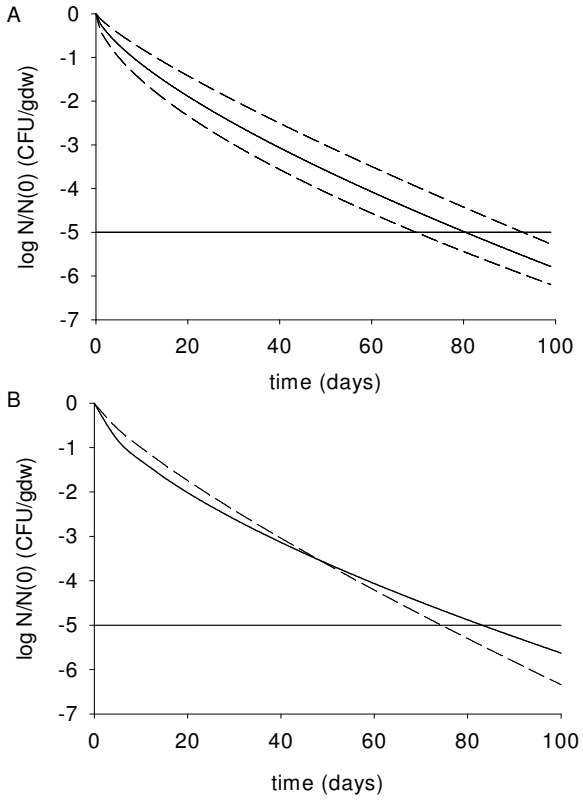

Fig. 3. Average Weibull decline curve of survival of *E. coli* O157:H7 in A) all 36 manure-amended soils (solid) ± 1 standard deviation (dotted) and B) in sand (solid) versus loam (dotted).

significant effect of soil type on parameters *b* and *n* was present within both ORG and CONV soils. Soil management (ORG versus CONV) did not show an overall effect on the combined variance of the three parameters (p=0.109, Wilks Lambda = 0.815). The first decimal reduction time was significantly lower for ORG sandy soils compared to CONV loamy soils (p<0.0001) and lower (but just not significant) for ORG loam compared to CONV loam (p=0.061) (Fig. 4). No effects of management were found on parameter *n* and *ttd*.

Differences in soil characteristics between soil type and management regime. The *pH* and *copio/oligo* ratio were significantly higher for loamy soils (both p<0.0001) while the NH_4 content was higher (p<0.0001) for sandy soils, especially in CONV sandy soils. None of the measured soil characteristics differed significantly between ORG and CONV manure-amended soils, although the microbial species richness *S* tended to be higher in ORG sand compared to CONV sand (p=0.064). This microbial species richness *S* was positively correlated with the number of years of certified

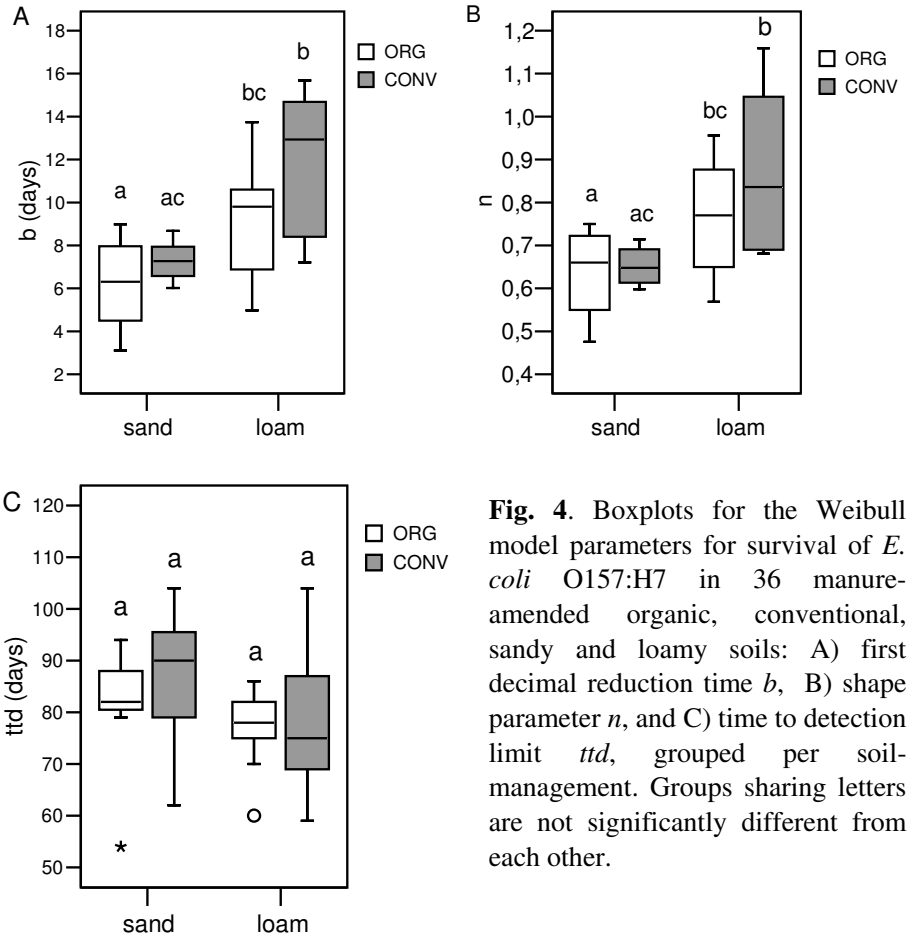

Fig. 4. Boxplots for the Weibull model parameters for survival of *E. coli* O157:H7 in 36 manure-amended organic, conventional, sandy and loamy soils: A) first decimal reduction time *b*, B) shape parameter *n*, and C) time to detection limit *ttd*, grouped per soil-management. Groups sharing letters are not significantly different from each other.

organic management (r=0.34, p=0.045), indicating a higher species richness with increasing years of organic management.

Relation between survival and manure-amended soil characteristics. Considering all 36 soils, the time needed to reach the detection limit *ttd* showed a positive correlation with the level of dissolved organic carbon [*DOC*] and the DOC per unit of biomass [*DOC/biomC*] (Table 1, Fig. 5). Also within sandy soils the *ttd* was positively correlated with *DOC/biomC*. The *ttd* of loamy soils was positively correlated with *DOC/biomC*, CO_2*/biomC* and negatively with *biomass* and *sand*. Within CONV soils the *ttd* positively correlated with *DOC/biomC* and NO_3 and negatively with *pH*. In

ORG soils *ttd* was significantly positively correlated with *DOC* and *DON* but only at the 0.1 significance level with *DOC/biomC*.

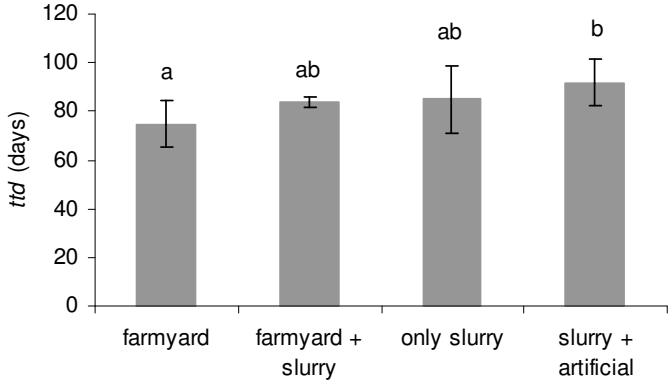

Fig. 5. Survival time of *E. coli* O157:H7 in manure-amended soil per type of manure management history of the original production field. Groups sharing the same number do not differ significantly from each other.

Multiple regression analysis revealed a uniform picture with respect to identification of factors most explaining the variation in survival times (Table 2). The *ttd* within all 36 soils as well as within sandy soils, loamy soils (after excluding the percentage of sand particles as a variable) and CONV soils was best explained by the *DOC/biomC*. In all cases the relation between *ttd* and *DOC/biomC* was positive. The remaining variation in *ttd* over all 36 soils was best explained by the ammonium content (NH_4, positive relation) and the number of years under organic management (*years*, negative relation). Within loamy soils the variation in *ttd* was best explained by the physical composition of the soil, i.e. the fraction of sand particles relative to clay particles (negative relation). When excluding *sand*, *DOC/biomC* was also the main determinant of *ttd* within CONV soils. The remaining variation was best explained by *biomass* (negative relation). Within ORG soils *DOC* and *DON* were the best predictors for *ttd* (positive relations). Remaining variation could be explained by microbial species diversity *H* (negative relation).

Over all 36 soils the *DOC/biomC* was negatively correlated with the percentage of sand particles (*sand*) and positively correlated with the microbial activity per unit biomass (CO_2*/biomC*). In addition, over all 36 soils as well as within the separate groups (sand, loam, ORG, CONV) the *DOC/biomC* was positively correlated with the moisture content (*moist*).

Table 1. Pearson correlations between the time needed to reach the detection limit (*ttd*) and manure-amended soil characteristics.

		sand	*DOC*	*DON*	*biomass*	*DOC/biomC*	*CO_2/biomC*	*NO_3*	*pH*
All samples	*ttd*	ns	+ 0.33*	+ 0.30	ns	+ 0.52*	ns	ns	ns
Sand	*ttd*	ns	ns	ns	ns	+ 0.75*	ns	ns	ns
Loam	*ttd*	- 0.54*	ns	ns	- 0.44*	+ 0.46*	+ 0.45*	ns	ns
ORG	*ttd*	ns	+ 0.58*	+ 0.63*	ns	+ 0.36	ns	ns	ns
CONV	*ttd*	ns	ns	ns	ns	+ 0.64*	ns	+ 0.52*	- 0.49*

ns: no significant correlation, no asterisk: correlation at the 0.1 level (2-tailed), * significant at the 0.05 level (2-tailed)

Table 2. Best regression models for the time needed to reach the detection limit *ttd* (based on the Weibull decline model) for *E. coli* O157:H7 in 36 manure-amended soils (organic/conventional, sand/loam), based on 16 different abiotic and biotic characteristics of the manure-amended soils.

Group	Model *ttd* =	p-value	R^2
Overall	$46.49^{***}(\pm 8.14) + 20.03^{***}(\pm 4.54) \times \boldsymbol{DOC/biomC} + 13.49^{**}(\pm 4.92) \times \boldsymbol{NH_4} - 0.56^{*}(\pm 0.34) \times \boldsymbol{years}$	<0.001	0.45
Sand	$60.10^{***}(\pm 6.21) + 22.57^{***}(\pm 5.51) \times \boldsymbol{DOC/biomC}$	0.001	0.57
Loam	$78.19^{***}(\pm 12.15) - 0.44^{***}(\pm 0.15) \times \boldsymbol{sand} + 3.98^{**}(\pm 1.77) + 11.49^{*}(\pm 6.56) \times \boldsymbol{DOC/biomC}$	0.005	0.46
	$77.11^{***}(\pm 16.36) + 12.93^{**}(\pm 7.99) \times \boldsymbol{DOC/biomC} - 6.53^{*}(\pm 4.26) \times \boldsymbol{biomass}$	0.043	0.30
ORG [a]	$47.62^{***}(\pm 19.24) + 43.57^{***}(\pm 6.76) \times \boldsymbol{DON} - 20.09^{**}(\pm 9.93) \times \boldsymbol{H}$	<0.001	0.69
CONV [b]	$127.65^{***}(\pm 23.50) + 22.72^{***}(\pm 5.51) \times \boldsymbol{DOC/biomC} - 57.15^{***}(\pm 18.02) \times \boldsymbol{pH}$	0.001	0.66

* p<0.15, ** p<0.10, *** p<0.05.

[a] Alternative model: $ttd = -9.11\ (\pm 14.53) + 48.52^{***}(\pm 8.08) \times \boldsymbol{DOC} - 18.86^{**}(\pm 10.41) \times \boldsymbol{H}$, p<0.001, R^2=0.67.

[b] Alternative model: $ttd = 126.02^{***}(\pm 27.91) + 14.48^{***}(\pm 5.67) \times \boldsymbol{NO_3} - 55.26^{***}(\pm 20.99) \times \boldsymbol{pH} + 7.73^{**}(\pm 3.83) \times CO_2/biomC$, p=0.009, R^2=0.58.

Extremes and management history. Although on average no differences in overall survival time of *E. coli* O157:H7 were found between soils with different management and between different soil types, some interesting differences were detected between the four best (*ttd* of 104, 104, 98 and 92 days) and four worst supporting soils (*ttd* of 54, 59, 60 and 62 days). The four most supporting soils were all CONV soils (three sand, one loam) and showed a significantly higher value for shape parameter *n* ($p=0.03$), *ttd* ($p<0.001$), NO_3 ($p=0.048$) and *DOC/biomC* ($p=0.040$). The four worst supporting soils included two ORG and two CONV soils (one sand and loam each).

The four soils with slowest decline of *E. coli* O157:H7 all had a history of slurry and artificial fertilizer application (three only slurry and one slurry with additional artificial fertilizer) while three of the four soils with the fastest decline generally received manure with a higher C/N ratio (two received a mixture of slurry and farmyard manure, one only composted farmyard manure and one only slurry). This relation between pathogen survival and the history of manure type application to the fields was also visible within the whole set of 36 soils. In general the decline of *E. coli* O157:H7 was increasing with increasing history of solid manure with relatively high C/N ratio (Fig. 6).

Discussion

Performance of the Weibull model. The Weibull model proved to be a suitable model for describing the decline of *E. coli* O157:H7 in manure-amended soil. The model is sufficiently flexible to account for different survival patterns (linear when $n=1$, concave when $n>1$ and convex when $n<1$) and has been previously used to model thermal pathogen inactivation (van Boekel, 2002) and the survival of soilborne plant pathogens (Shlevin et al., 2003). An additional advantage of the Weibull model is the absence of a lower asymptote which can overestimate the survival over prolonged periods (Coroller et al., 2006). With the present study the shape parameter *n* was different from 1 in all but two cases, which indicates that the use of first-order decline models to describe the survival of *E. coli* O157:H7 in manure-amended soil is not justified. Even though the Weibull model is an empirical model, it can be linked to physiological properties at population level. The model is based on the assumption that the population is heterogeneous with respect to the stress encountered in the substrate. This variability in stress is assumed to follow a Weibull distribution and therefore the survival over time can be modelled with the cumulative form of the distribution. A convex curve ($n > 1$) would mean that the remaining cells become increasingly susceptible to stress, which can be interpreted as evidence that accumulated damage weakens the survivors (and hence their destruction rate will increase with time) (van Boekel, 2002; Peleg, 2003). A linear semi-logarithmic survival curve ($n = 1$) means that the probability of dying does not depend on time, i.e. there is no effect of accumulated damage and/or there is no biological variation in the pathogen population,

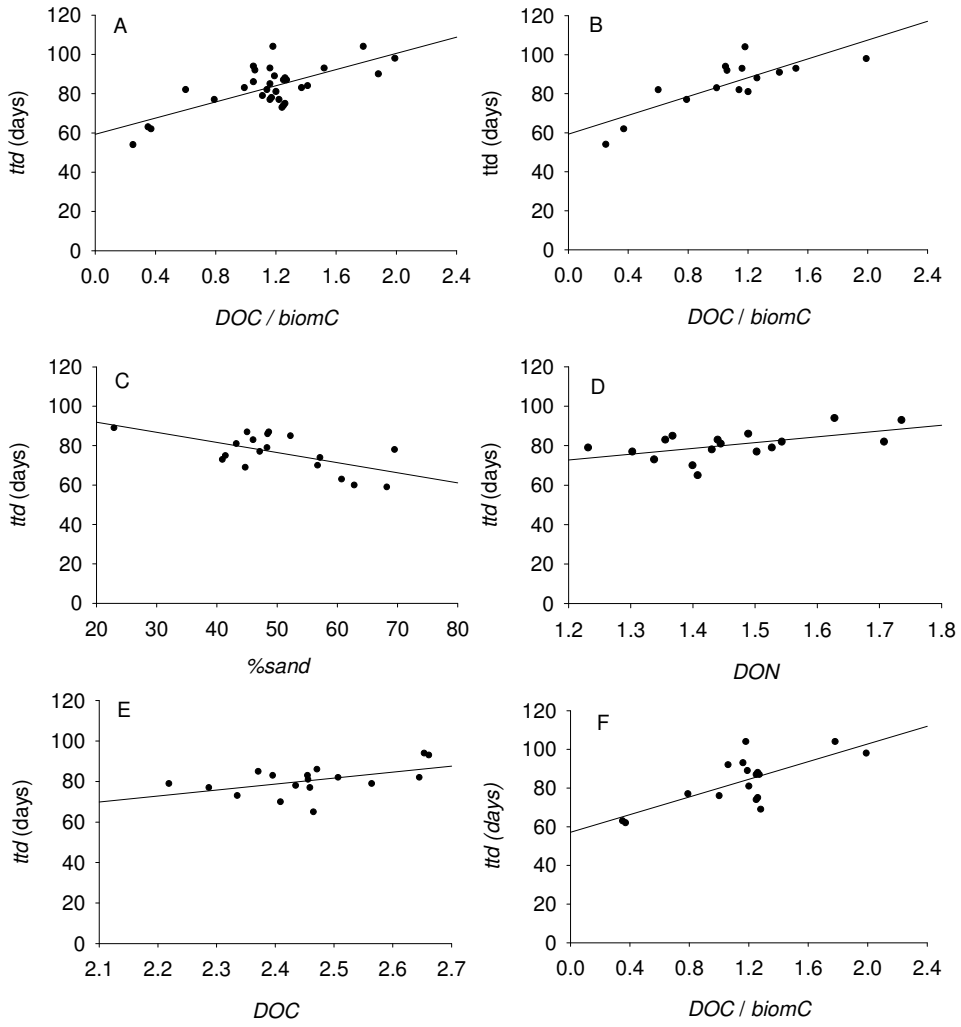

Fig. 6. Scatterplots and correlations between the time needed to reach the detection limit (*ttd*) and A) *DOC/biomC* (unitless log value) in all 36 soils, B) *DOC/biomC* (unitless log value) in the sandy soils, C) *sand* (%) in the loamy soils, D) *DON* (log mg/kg) in the organic soils, E) DOC (log mg/kg) in organic soils and F) *DOC/biomC* (unitless log value) in the conventional soils.

antagonistic microbial community or predatory activity. In the present study, the average value of n is smaller than 1 (0.74), which resulted in a concave curve. This can be interpreted as evidence that sensitive members of the population are rapidly

eliminated and that the sturdier survivors remain (Peleg, 2003). This might be the result of intrinsic biological variation within the population that is already present at the start of the exposure to stress, or of the variation evolving during the exposure time because cells adapt to the encountered stress (van Boekel, 2002). Alternatively, the antagonistic microbial community or predatory fauna could change over time, resulting in decreasing stress for the surviving cells of *E. coli* O157:H7. Since we obtained different survival curves while using one single inoculum culture and one identical inoculum carrier, it is most likely that changes in (biological) soil characteristics determine whether cells can survive and to what extent and rate. Different soils will react differently to the addition of manure, which results in different nutrient conditions (as expressed by e.g. *DOC/biomC*), microflora and fauna and thereby different survival patterns for *E. coli* O157:H7.

Survival time. The main objective of this study was to assess the variability in decline kinetics of *E. coli* O157:H7 in a range of different soils and to identify critical factors that determine the rate of decline. Although comparison with other studies is difficult because of the use of different experimental setups and different statistical analyses, the range of survival times (54 to 105 days, average 80) determined in the present study corresponds to those published earlier (Bolton et al., 1999; Maule, 2000; Mubiru et al., 2000; Vinten et al., 2002). The determination of *E. coli* O157:H7 survival in 36 soils allowed for the quantification of the variability in overall survival times, which followed a logistic distribution. The shape of this distribution indicates that the chances of a longer and a shorter than average survival are approximately equal. The probability distributions of the model parameters and/or the overall survival times for the survival of *E. coli* O157:H7 in manure-amended soils can be used to increase the accuracy of exposure assessments of vegetables or water contamination.

Difference between soil types and management regimes. With the present study we found that the values of the log reduction time b and the shape parameter n of the Weibull model were significantly higher for loamy soils compared to sandy soils. This means that with sandy soils the initial rate of decline of *E. coli* O157:H7 is faster but that the decline rate slows down more with progressing time than with loamy soils. Apparently, *E. coli* O157:H7 is more vulnerable to mortality during the first 1-2 weeks in the sandy manure-amended habitat but survivors are increasingly more sturdy compared to survival in the loamy soils. As a result, average survival curves of sandy and loamy soils crossed and no difference was observed in overall survival time between sandy and loamy soils. However, within loamy soils the survival of *E. coli* O157:H7 was primarily determined by the soil texture, with longer survival associated with relatively more clay particles compared to sand particles. Survival of *E. coli* O157:H7 has been reported to be prolonged in finer textured soils (Fenlon et al., 2000;

Mubiru et al., 2000; Nicholson et al., 2005). However, most published data on the survival of *E. coli* O157:H7 in soil typically included only a limited number of different soils, which does not fully justify generalized conclusions on the effect of soil type. In contrast, the present study included 36 different soils and showed a faster initial decline of *E. coli* O157:H7 in sandy soils but no differences in the final survival time between both soil types.

In general it is stated that finer textured (clayey) soils result in prolonged survival of introduced bacteria compared to coarser textured (sandy) soils because of higher availability of protective pore spaces against feeding by soil fauna like protozoa (van Veen et al., 1997). This could explain the faster initial decrease in *E. coli* O157:H7 numbers in the sandy soils compared to the loamy soils. But for reasons which are unclear at the moment the decline rate in sandy soils decreases with time relative to the decline in loamy soils. A possible explanation might be that the presence of manure decreased the attachment of fecal coliforms to clay and silt fractions to a higher extent than with sand fractions (Guber et al., 2007). In addition, with small pore spaces soil moisture and dissolved organic matter are more difficult accessible for microbes (Marschner and Kalbitz, 2003). Higher clay content was associated with a more negative matrix potential (and thus higher water stress) and a faster decline of *E. coli* (Mubiru et al., 2000). In the present study we attempted to minimize the possible overruling effect of soil moisture availability by adjusting the soil moisture content to 60% of the water holding capacity. The gravimetric water content did not differ between both soil types during the experiment but the matric potential was not measured. However, survival of *Pseudomonas fluorescence* and *E. coli* O157:H7 was found to be unaffected by the matric potential (Meikle et al., 1995; Ritchie et al., 2003).

No differences in survival of *E. coli* O157:H7 were observed between the soils of both management regimes. This is probably related to the absence of differences in chemical and biological soil characteristics between both management regimes. In contrast, earlier data on 12 soil pairs which were also included in the present study showed significantly higher levels of NO_3, basal respiration rate, numbers of copiotrophic and oligotrophic bacteria, microbial species richness (not the diversity) and nematode diversity in the organically managed soils (van Diepeningen et al., 2006). In the present study, microbial species richness and diversity were higher in organic soils but these differences were not significant. This might be the result of the addition of the same manure as inoculum carrier to all soils and the disturbance of the soil microbial community by sampling and mixing.

Influence of chemical and biological soil characteristics. The single soil property that appeared to be the best predictor of *E. coli* O157:H7 survival in manure-amended soil is the level of dissolved organic carbon per unit of biomass carbon (*DOC/biomC*),

i.e. the level of easily available carbon sources per unit of soil biomass. Additional positive relations were found between survival and levels of *DOC*, *DON* and *CO_2/biomC*. When *E. coli* leaves its primary habitat, the nutrient-rich (copiotrophic) anaerobic intestine of warm-blooded animals with an ample supply of carbonaceous compounds (Macfarlane and Macfarlane, 1997), it has to adapt to its nutrient-deficient (oligotrophic) and largely aerobic secondary habitat like soil where the concentration of energy sources is usually very low (Hattori and Hattori, 1976). Although *E. coli* can potentially exhibit oligotrophic kinetic properties in chemostat cultures (Kovarova-Kovar and Egli, 1998), a major factor in *E. coli* die-out in soils is thought to be its inability to lower its metabolic rate to meet the low availability of usable organic carbon and to adjust to conditions of low nutrient availability (Klein and Casida, 1967; Jamieson et al., 2002). With the present study we showed that the survival of the copiotrophic *E. coli* O157:H7 in manure-amended soil is indeed longer in more copiotrophic (more *DOC, DON, DOC/biomC* and *CO_2/biomC*) manure-amended soil systems. In addition, increased levels of easily available energy sources in DOC may (temporarily) decrease the competitive pressure between organisms and thus possibly allow increased persistence.

Within ORG soils survival of *E. coli* O157:H7 was predominantly determined by the absolute levels of dissolved organic carbon and nitrogen (*DOC* and *DON*). Although positively related, the *ttd* was not correlated with the *DOC/biomC*, like in CONV sandy, and loamy soils. The absence of *DOC/biomC* as an explanatory factor for the variability in *ttd* in the regression model for ORG soils is probably the result of the lower variability in *biomass* between the ORG soils compared to the CONV soils (standard deviation from mean respectively 0.49 and 0.66). In addition to the effects of *DOC* and *DON*, a negative correlation was found between the microbial diversity as measured by DGGE (*H*) was observed with a lower *ttd* with increasing *H*. Soils or rhizospheres with higher microbial diversity are expected to be more resistant to stress and disturbances and consequently less susceptible to invasion (Kennedy et al., 2002; Lynch et al., 2004; Matos et al., 2005). The results of the present study indicate a potential importance of an increased microbial diversity in managing the survival of human pathogens.

Within CONV soils the survival time of *E. coli* O157:H7 was negatively related to the pH of the manure-amended soil. In contrast, several studies indicated a shorter survival of enteric bacteria in soils with lower pH (Gerba et al., 1975; Sjogren, 1994). However, it must be stressed that these studies all considered the survival of a generic *E. coli* strain and not a pathogenic strain like *E. coli* O157:H7. When grown in broth, pathogenic *E. coli* are significantly more acid tolerant than non-pathogenic strains (Gordon and Small, 1993). The virulence of *E. coli* O157:H7 is thought to be at least partially dependent on its ability to survive the low pH of the gastric stomach (Lin et al., 1996). Therefore, it is likely that *E. coli* O157:H7 has some selective advantage

over other bacteria at lower pH. This could also explain the decreasing decline rate with progressing time with the more acidic sandy soils compared to loamy soils.

Management factors to control the survival of *E. coli* O157:H7. Although no overall difference was found in *E. coli* O157:H7 survival in organic and conventional manure-amended soils, certain soil management aspects can be crucial to the control of human pathogens in soil systems. It should be realized that although we added the same amount of identical manure to each of the 36 soils, we found a considerable range within all measured chemical and biological variables in manure-amended soils. This means that levels of the measured variables depend on intrinsic soil characteristic and how the soil system reacts to the addition of manure.

The results of the present study indicate that the survival of *E. coli* O157:H7 is less prolonged under more oligotrophic conditions (i.e. lower levels of *DOC, DON* and *DOC/biomC*). Oligotrophication of agricultural ecosystems, which means the reduction of mineral nitrogen, soluble carbon compounds and available phosphorus, is thought to increase the natural suppression of plant diseases (van Bruggen and Termorshuizen, 2003). Such a soil system can be achieved by the regular addition of organic fertilizers characterized by a relatively high C/N ratio, like solid animal manure and compost of plant or animal origin to the soil (van Bruggen et al., 2006). Indeed, *E. coli* decreased more rapidly in soils treated with solid beef cattle manure compared to soils treated with liquid swine manure (Unc and Goss, 2006). In addition, soils with a high content of readily available nutrients showed a positive selection for α- and γ-proteobacteria, being indicative of r-selection, which is selection for bacteria with potentially high growth rates like *E. coli* O157:H7 (Smit et al., 2001). In low-nutrient soil or soil with a high content of recalcitrant substrates, the percentage of *Acidobacterium* increased, being indicative of k-selection, which is selection for bacteria with lower growth potential but higher capability to compete for substrates.

Improvement of manure quality receives increased attention in order to reduce ammonia emission, nitrate leaching, stimulate microbial activity in the soil and increase soil organic nitrogen content (Reijs et al., 2007). This can be achieved by increasing the C/N ratio of the manure, which in turn can be achieved by feeding cattle a diet with a higher fibre content or mixing manure with straw. In addition, *E. coli* O157:H7 has been found to decline significantly faster in manure derived from cattle which were fed high fibre diet compared to a low fibre diet (Franz et al., 2005). When applied to soil, this results in a relatively slow release of easily available nitrogen and carbon sources and increased net nitrogen immobilization in the soil. This creates a more oligotrophic system which is a disadvantage for the copiotrophic *E. coli* O157:H7. Fertilizer of low quality (like slurry or artificial fertilizer) would have the opposite effect since this is relatively rich in readily available carbon and nitrogen sources, which in turn can lead to eutrophication of the soil. The application of liquid

manure to soil was found to bring triple the amount of water-extractable organic carbon compared to the application of solid manure (Angers et al., 2006). Moreover, urea-based and ammonium-based fertilizers temporarily solubilise soil organic matter and can induce a marked increase in dissolved organic carbon content (Chantigny, 2003). An eutrophicated soil system will not only be subjected to higher nutrient losses (Reijs et al., 2007) and lower natural suppression of plant pathogens (van Bruggen et al., 2006) but also to an increased survival of human pathogens like *E. coli* O157:H7, which implies an increased risk of spread and transfer of this pathogen into the food chain.

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Chapter 5

Estimating the stability of *Escherichia coli* O157:H7 survival in manure amended soils with different management histories

Semenov, A.V., Franz, E., van Overbeek, L., Termorshuizen, A.J., and van Bruggen, A.H.C. (2008) Estimating the stability of *Escherichia coli* O157:H7 survival in manure amended soils with different management histories. *Environmental Microbiology* **10**: 1450–1459.

Abstract

The objective of this study was to describe survival of *E. coli* O157:H7 populations in manure-amended soils in terms of population stability, i.e. the temporal variation around the decline curve, in relation to soil characteristics indicative of soil health. Cow manure inoculated with *E. coli* O157:H7 was mixed with 18 pairs of organically and conventionally managed soils (10% of manure, kg/kg). For 4 of the soil pairs, also 3 different manure densities (5%, 10% and 20%) were compared. All soil-manure mixtures were incubated for two months, and population densities of *E. coli* O157:H7 were quantified weekly. De-trending of survival data was done by modified logistic regression. The residual values were used to assess variation in the changes of *E. coli* O157:H7 populations by performing the Approximate Entropy (ApEn) procedure. The term irregularity is used to describe this variation in ApEn literature. On average, the decline of *E. coli* O157:H7 was more irregular in conventional and loamy soils than in organic and sandy soils ($p<0.05$). Multiple regression analysis of irregularity of *E. coli* O157:H7 survival on 13 soil characteristics showed a positive relation with the ratio copiotrophic/oligotrophic bacteria, suggesting greater instability at higher available substrate concentrations. Incremental rates of manure application significantly changed the irregularity for conventional soils only. Estimation of temporal variation of enteropathogen populations by the Approximate Entropy procedure can increase the accuracy of predicted survival time and may form an important indication for soil health.

Introduction

High-quality, healthy soil is defined as a stable system with resilience to stress, high biological diversity, and high levels of internal cycling of nutrients (van Bruggen and Semenov, 2000). Generally a system is considered to be stable when it returns rapidly to the initial state or a new equilibrium with the least fluctuation and uncertainty (Holling, 1973; Botton *et al.*, 2006). With respect to soil, the ability to return to an equilibrium after stress has been studied only during the last few years. The influence of various disturbances (heavy metals, pH and organic or mineral amendments) on growth and population dynamics of bacterial communities have been measured to determine the resilience of the soil to these disturbances (Ellis *et al.*, 2003; Girvan *et al.*, 2005; Botton *et al.*, 2006; Tobor-Kaplon *et al.*, 2006; van Bruggen *et al.*, 2006). Microbial fluctuations in soils have been studied extensively in the past, but time series analysis to characterize temporal oscillations was carried out for few data sets only (Zelenev *et al.*, 2005). After a disturbance, microbial oscillations seem to dampen more quickly in healthy than in chronically damaged and biologically unbalanced soils (van Bruggen *et al.*, 2006). Quantitative measures for (in)stability of microbial populations in soils have not been suggested so far.

The standard deviation of a series of parameter values (e.g. population size of a certain species over time) is often used as a measure of temporal variability (McGrady-Steed *et al.*, 1997). However, this measure can overestimate the true temporal variation by not accounting for the presence of a specific pattern of variation (e.g. oscillation) of a population (Gould and Nichols, 1998). Recently, a mathematical approach, referred to as Approximate Entropy (ApEn), was introduced to quantify serial irregularity (Pincus, 1991). This mathematical approach reflects the likelihood that certain patterns of observations will not be followed by additional similar observations. ApEn assesses temporal variation on a continuum ranging from totally regular (specific pattern without uncertainty) to maximally irregular (completely random). ApEn is nearly unaffected by noise, is robust and insensitive to artifacts: extremely large and small outliers have only little effect on the ApEn calculation. Quantitative assessments of irregularity have been performed for price changes in stock markets (Pincus and Kalman, 2004) and human physiology (Richman and Moorman, 2000). ApEn analysis is an appropriate tool to characterize the extent of irregularity, while the standard deviation can only estimate the extent of deviation from centrality (Pincus and Kalman, 2004). Moreover, irregularity calculated by ApEn analysis can be applied as an indicator of system stability: relatively high ApEn values may indicate unpredictable variation in a system (Pincus, 1991). Thus, a population density time series of a certain microorganism introduced into a natural substrate can result in an appropriate assessment of the irregularity of its behavior and possibly the stability (and health) of the system.

Growth and survival of invasive populations of microorganisms are major ecological processes which are important for temporal changes in the structure of autochthonous microbial communities, intensity of competition, food webs and nutrient cycles in natural substrates such as soil, manure and compost (van Veen *et al.*, 1997). The spread of human pathogenic bacteria in the environment and food chain can be considered as an evident example of invasion by microorganisms. For pathogens with animal reservoirs such as *E. coli* O157:H7, contamination of vegetables in the field can occur when contaminated manure is used as fertilizer. Survival of *E. coli* O157:H7 in different substrates such as soil, manure and soil-manure mixtures can range from several weeks to more than a year (Kudva *et al.*, 1998; Nicholson *et al.*, 2005). Once enteropathogens are introduced into soil, they start to interact with the autochthonous microbial community. Microbial soil properties and their dynamics depend on abiotic characteristics, organic and mineral amendments (Marschner *et al.*, 2003; Stark *et al.*, 2007), availability of C and N fractions (Hu et al., 1999; Landgraf and Klose, 2002) and biotic interactions.

Soil management practices can also influence the microbial community (van Diepeningen *et al.*, 2006; Fließbach *et al.*, 2007) and therefore the behavior of *E. coli* O157:H7 populations in soil. Conventional systems are characterized by intensive farming procedures, application of artificial fertilizers, herbicides etc. High levels of stress decrease microbial diversity due to lack of sufficient tolerance of a range of species to particular stress factors (Giller *et al.*, 1998). Generally, organically managed soils show a higher diversity of bacteria and higher microbial biomass than conventionally managed soils (Mader *et al.*, 2002; van Diepeningen *et al.*, 2006). Such characteristics make them less susceptible to invasive pathogens and increase resilience and resistance to stress (van Bruggen and Semenov, 2000). Estimation of dynamic parameter changes (e.g., populations of introduced bacteria) in different types of soil can assist in assessing soil stability. In the case of human pathogens, a periodical (predictable) pattern around the mean of a survival curve, even with large amplitude is more preferable for assessment and minimization of risk of pathogen survival in agricultural systems than irregular (unpredictable) changes. Knowledge of enteropathogen stability in soil can be directly used in a risk assessments as well as a characterization of soil health.

In the present study, survival of *E. coli* O157:H7 was determined over time in 18 pairs of organically and conventionally managed soils; 12 of these pairs had been analyzed for several soil health parameters previously (van Diepeningen *et al.*, 2006). The objectives were (i) to characterize the irregularity of *E. coli* O157:H7 survival in soils amended with manure by performing approximate entropy analysis, (ii) to determine potential differences in irregularity of *E. coli* O157:H7 survival between management regimes and soil types and (iii) to determine chemical, physical and biological manure-amended soil characteristics which are associated with differences

in temporal variability and uncertainty of survival among soils. Part of the data set has been previously analyzed to compare differences in survival rates between management and texture types of soils mixed with manure (Franz *et al.*, 2008). The average decline rates did not significantly differ among manure-amended soils from various origins.

Materials and Methods

Soil and manure collection. Soil was collected from six organic farms with lettuce fields throughout the Netherlands. Six conventional farms with lettuce fields were selected on comparable soil types as close as possible. The nearest set of farms was considered as a pair. Distance between pairs varied between 1 or 2 km. In addition, 12 organic farms with other crops were selected throughout the Netherlands as described in detail by (van Diepeningen *et al.*, 2006). For these 12 organic farms, neighbouring conventional farms were selected by choosing an adjacent field with the same soil type and with the same crop (potato, grassland, sugar beet, wheat or maize). Each pair of organic and conventional farms was sampled on the same day. Throughout each sampled field, 10 soil sub-samples (diameter 30 cm, 20 cm deep) were collected between the plants and mixed. All samples were collected in April and May 2005. All samples were transported to the laboratory in plastic bags, thoroughly mixed, sieved through 0.5 cm mesh to remove plant parts and earthworms and stored at 5 °C until the start of the experiment (May 2005).

Fresh manure without urine from organically managed Holstein Frisian steers on a standard 50% grass/clover-silage + 50% dried grass diet was mixed with straw (90% manure and 10% straw (kg/kg, dry weight) and stored for nearly one month in a heap (sampling depth 20cm, 30-40 °C at that depth) at the organic experimental farm Droevendaal (Wageningen University and Research Center, Wageningen, The Netherlands). About 10 kg of this manure was collected from the heap in May 2005, homogenized and stored in closed plastic bags at 5 °C for two weeks. Seventy-two portions of 50 g were weighted and put in small plastic bags.

Soil texture. Fractions of clay particles (<2 μm), silt particles (2-50 μm) and sand particles (50-2000 μm) were determined in all collected soils by laser diffraction before mixing with manure. Soils were classified into soil types according to a US texture diagram (Saxton *et al.*, 1986) and summarized as being sand or loam.

Adjusting water content. To ensure similar water availability in the different soils the water content of each individual soil was adjusted to 60% of its maximum water holding capacity (WHC). Subsequently, two portions of 450 g from each soil were placed in plastic bags. One portion was mixed with non-inoculated manure and used as a control on which all chemical and biological characteristics were determined (all at

the start of the experiment) and one portion was mixed with inoculated manure to study pathogen survival.

Chemical characterization. Dried (24 h at 40 °C) soil-manure samples from the control pots were ground, sieved through a 2 mm mesh and analyzed at the start of the experiment. The pH was measured in water with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3^-), ammonium (NH_4^+) and total dissolved nitrogen (N_d) content were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic nitrogen (DON) was calculated as the difference between N_{ts} and the amount of nitrogen present as NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Total carbon (C_{total}) and total nitrogen (N_{total}) were measured by the Dumas method followed by detection by a Fisons element analyzer type EA 1108 (Therom Finnigan Italia S.P.A., Milan, Italy). Details for chemical measurements are provided in (Franz *et al.*, 2008).

Biological characterization. Microbial activity was assessed by measuring the basal respiration rate. Microbial carbon present in the manure-amended soils was measured using the fumigation extraction method (Vance *et al.*, 1987). DGGE (Denaturing Gradient Gel Electrophoresis) analysis was conducted on eubacterial DNA extracted directly from soil-manure mixtures in order to relate the survival of *E. coli* O157:H7 to the species richness and species diversity of the microbial population present in the manure-amended soils. The bacterial diversity was estimated in two ways: as species richness *S*, and as the Shannon-Wiener index of bacterial diversity, *H*. Species richness *S* was defined as the number of DGGE detected bands per soil type. The Shannon-Wiener diversity index was calculated as $H' = -P_i \log P_i$ based on the relative band intensities as formulated by Eichner *et al.* (Eichner *et al.*, 1999), where P_i (importance probability of the band) is defined as n_i/N where n_i is the area of the peak in intensity and N the sum of all peak areas in the lane profile. The Shannon-Wiener diversity index is a general diversity index which increases with the number of species and which is higher when the mass is distributed more evenly over the species. Moreover, number of copiotrophic and oligotrophic bacteria was quantified by counting colony forming units (CFUs) on C-rich and C-poor media, respectively. Details for biological measurements are provided in (Franz *et al.*, 2008).

Strain. *E. coli* O157:H7 strain B6-914 *gfp*-91 was provided by P. Fratamico (Fratamico *et al.*, 1997). The strain had been modified from strain SEA 13B88 (from the outbreak linked to Odwalla apple cider; Food and Drug Administration), so that it contained green fluorescent protein (*gfp*) (pGFP cDNA vector) and ampicillin resistance, while the Shiga-like toxins ($Stx1^-$ and $Stx2^-$) were deleted. These changes

did not result in any significant differences in survival in nutrient media compared to the wild-type strain (Fratamico *et al.*, 1997; Vialette *et al.*, 2004). Green fluorescence of *gfp*-transformed strain was checked under UV light. Stock cultures were stored in 30% (w/w) glycerol at -80 °C.

Inoculation of manure and preparation of soil-manure mixture. Bacterial inocula were grown in Erlenmeyer flasks containing 150 ml fresh Luria-Bertani broth, with 50 μg ml^{-1} ampicillin for *E. coli* O157:H7 (Sigma-Aldrich Chemie Gmbh, Germany), followed by incubation at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The number of cells ml^{-1} of suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm in 1 ml cuvet was equal to 1×10^9 CFU ml^{-1}. Prepared inocula were added by a pipette to manure and mixed thoroughly within a double layer of plastic autoclavable bags. After thorough kneading of plastic bags by hand for 5 min., 50 g of the inoculated manure was mixed with 450 g of each soil with a final density of 10^7 CFU per gram of soil-manure mixture dry weight (gdw^{-1}). The concentration of manure was 10% (kg kg^{-1}) for 18 pairs of organically and conventionally managed soils. Additionally, 4 pairs of soil with 3 different concentrations of manure (5%, 10% and 20%) were prepared. All soil-manure mixtures inoculated with *E. coli* O157:H7 were incubated for 60 days at 16 ºC in plastic pots.

Sampling procedure of soil-manure mixtures. Two samples for every soil amended with manure, approximately 0.5 g of fresh sample, were put in pre-weighed dilution tubes with 4.5 ml of 0.1% peptone buffer to determine the exact weight. Samples were vortexed and sonicated for 30 s (Branson 5200, 120-W output power, 47 kHz). Ten-fold dilution series were prepared with 0.1% peptone buffer, and 50 μl of the two highest dilutions per sample was plated in duplicate on sorbitol-MacConkey agar (Oxoid) with ampicillin (50 μg ml^{-1}). After adding approximately 20 sterile glass beads per Petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass beads to spread the inoculum over the surface of the plate. Fluorescent bacterial colonies were counted under a UV lamp (365 nm UV-A, PL-S, Philips, Eindhoven, the Netherlands) after incubation at 37 °C for 24 h. Fluorescent colonies made up 95-99% of all colonies on a plate. Fluorescent colony-forming units (CFU) were calculated per gram of dry soil-manure mixture. Samplings were done two times per week during first month and weekly during second month. The calculated detection limit was 10 CFU gdw^{-1}.

Calculation of residuals. Number of colony forming units (CFU) for each Petri dish was transformed to CFU per gram of dry weight (gdw^{-1}). To calculate the residuals for

decline of *E. coli* O157:H7 in each soil-manure mixture, log-transformed data were fitted to the modified logistic function (Zwietering *et al.*, 1990) by nonlinear regression (Gauss-Newton method) (SAS version 8.02, SAS Institute Inc., Cary, USA, 2001): $C_t = a / [b + \exp(d \times (t - m))]$; where C_t is the log CFU gdw^{-1} at time t (days), $(a + b)$ is the upper asymptote (CFU gdw^{-1}), b is the lower asymptote (CFU gdw^{-1}), m is the position parameter (referred to as point of 50% of reduction) and d is the slope parameter for the decline rate (days^{-1}). The upper and lower asymptotes were kept constant at 6.5 log CFU gdw^{-1} and 0, respectively. Samples which gave a CFU count of 0 were included in the analysis with the value of 1 log CFU gdw^{-1} which was the detection limit. Parameters and residuals for the survival curves were estimated with the NLREG program (version 6.3, P. H. Sherrod, 2005). The significance and fit of the estimated decline rates were assessed by the F value of the non-linear regression and the non-linear coefficient of determination (pseudo-R^2) for each curve, respectively (Fig. 1). Sampling errors due to spatial variation and analysis procedure were calculated as standard error (standard deviation / square root (N)) for each sampling time of every soil-manure mixture. Calculated sampling errors were subtracted from the residuals. If a particular residual was less than the calculated sampling error then the residual was assigned as zero (Fig. 2). Furthermore, the data were transformed to be equidistant. The linear spline interpolation procedure was used to estimate one missing value in the data set.

Estimating irregularity for *E. coli* O157:H7 population. The approximate entropy (ApEn) method was used to quantify serial irregularity (Pincus, 1991). Formally, given N points, ApEn(m, r, N) is approximately equal to the negative average natural logarithm of the conditional probability that two sequences that are similar for m points remain similar within a tolerance window r (which defines the criterion of similarity), at the next point. ApEn(m, r) was calculated according to the formula of Pincus (Pincus, 1991) with fixed input variables $m = 1$ and $r = 20\%$ of the SD of the specified time series. A time series containing many repetitive similar patterns (less irregular) has a relatively small ApEn; a less predictable (more complex and more irregular) process has a higher ApEn (Pincus, 1991). A technical discussion of mathematical and statistical properties of ApEn can be found elsewhere (Pincus and Goldberger, 1994). Irregularity for *E. coli* O157:H7 survival was calculated with the Pulse_XP software package (M. L. Johnson, University of Virginia, 2004).

Statistical analysis. Differences in irregularity of *E. coli* O157:H7 survival between management types, soil types, as well as among manure concentrations were calculated by two-sided t-tests. Correlation tests were performed to check for linear relationships between irregularity and biological/chemical parameters of the soil-manure mixtures (SPSS version 12, SPSS Inc., Chicago, Illinois, USA, 2003). Stepwise multiple

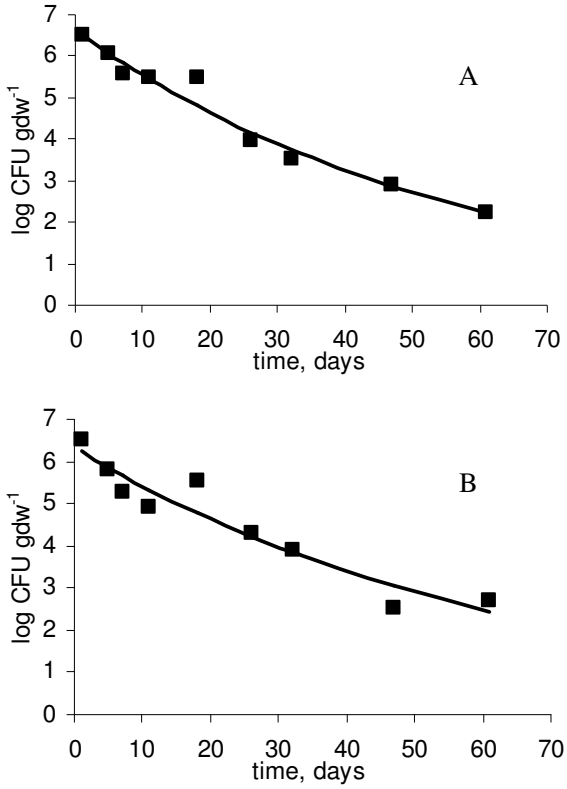

Fig. 1. Representative examples of fitting observed values of *E. coli* O157:H7 survival at 16 ºC (closed squares) by a modified logistic model (solid lines) for organically (A) and conventionally (B) managed soils amended with manure.

regressions were conducted by the REG procedure (SAS version 8.02, SAS Institute Inc., Cary, USA, 2001) to determine to what extent variation of biological/chemical parameters can explain variation in irregularity. Regression analyses were conducted on normalized data to avoid possible nonlinear relations. The following parameters were included in the analysis: irregularity of *E. coli* O157:H7 survival (*irregularity*) calculated by the ApEn procedure, percentage of sand fraction (50-2000 μm) in soil (*sand*), years being organically certified (*years*), microbial diversity as determined by DGGE (*H*), species richness as determined by DGGE (*S*), log (nitrate in mg kg^{-1} dw^{-1}) (NO_3), log (ratio inorganic nitrogen over organic nitrogen in mg/mg) ($N_{in}N_{or}$), log (C/N ratio in mg mg^{-1}) (*C/N*), log (ratio dissolved organic carbon over dissolved organic nitrogen in mg mg^{-1}) (*DOC/DON*), log (ratio number of copiotrophic bacteria over number of oligotrophic bacteria) (*copio/oligo*), log (moisture content in percentage of total weight) (*moist*), log (dissolved organic carbon per unit biomass carbon in mg mg^{-1} x 10) (*DOC/biomC*), log (basal respiration per unit biomass carbon in mg mg^{-1} kg^{-1} x 100) (CO_2/*biomC*) and log (ammonium in mg kg^{-1}) (NH_4). The pH was included after the following transformation: arsin(sqrt(pH/pH_{max})) (*pH*). The rate of manure

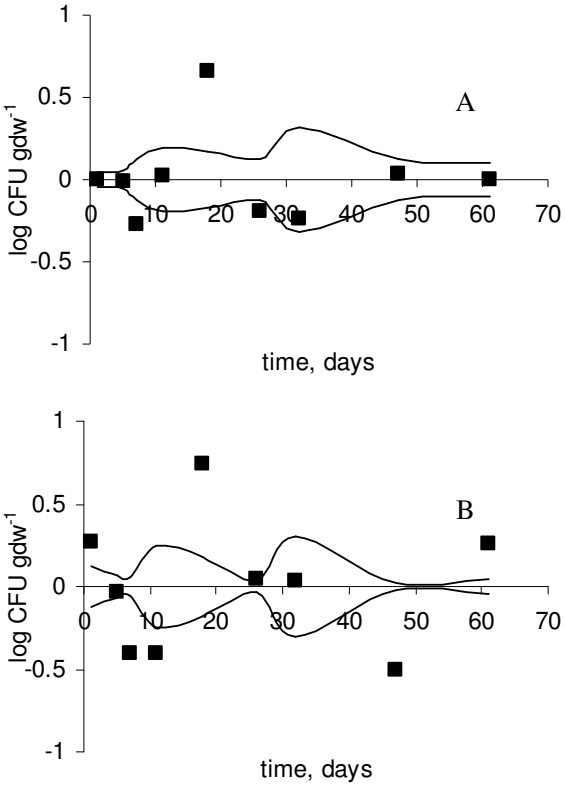

Fig. 2. Calculated residuals (closed squares) after de-trending by modified logistic model and sampling error (solid lines) for two *E. coli* O157:H7 decline curves for organically (A) and conventionally (B) managed soils amended with manure shown in Fig. 1.

application was included in the analysis as *rate*. Variables in the regression models were significant at the 0.1 level. Models were restricted to a maximum of two parameters. Multiple regressions were conducted for the overall dataset and for each management type and soil type separately.

Results

Immediately after inoculation, the average density of *E. coli* O157:H7 was 6.48 ± 0.04 log CFU gdw^{-1} for soil amended with manure. Pathogen populations significantly declined during the two-month incubation period in all 52 treatments of soil-manure mixtures (Fig. 1). Fitting of the survival data to the modified logistic function resulted in significant fits ($p<0.01$) for all soil-manure mixtures with an average pseudo-R^2 of 0.94 ± 0.04. No significant differences in R^2 and sampling errors between soil types and management types were found. Sampling errors were subtracted from the residuals to prevent the influence of spatial variation of samples and analysis procedure. In general, calculated residuals (on average 0.24 ± 0.02 log CFU gdw^{-1}) were significantly higher ($p<0.01$) than sampling errors (on average 0.10 ± 0.01 log

CFU gdw^{-1}) (Fig. 2). Only less than 15% of residuals were due to sampling error and were replaced by zero. The variability in residuals and sampling errors for *E. coli* O157:H7 survival in 36 soils amended with manure were represented by histograms (Fig. 3). The shape of distributions of sampling errors and residuals indicated that the majority of the samples were located at low ratios of sampling errors to residuals.

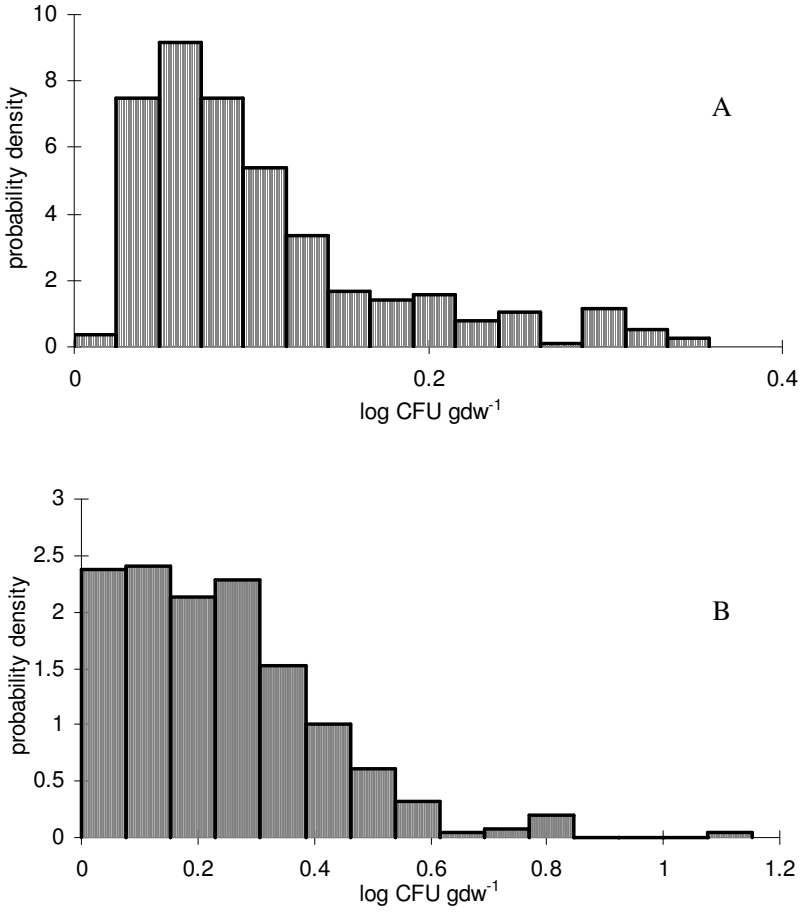

Fig. 3. Histograms of the sampling errors (A) and calculated residuals (B) after de-trending by modified logistic model for the survival of *E. coli* O157:H7 in 36 soils amended with manure.

Effect of soil type and management on characteristics of manure-amended soils and irregularity in *E. coli* O157:H7 survival. The *pH* and *copio/oligo* ratio were significantly higher in loamy soils ($p<0.001$) compared to sandy soils. The NH_4 content

was higher ($p<0.001$) in sandy soils. No significant differences were found in chemical/biological soil characteristics between organically and conventionally managed manure-amended soils, although the microbial species richness *S* tended to be higher in organic compared conventional soils ($p<0.1$), despite the overwhelming effect of manure amendment.

On average, calculated *irregularity* for *E. coli* O157:H7 survival was 0.283 ± 0.178 (standard error of the mean = 0.025; confidence levels 95%: 0.233 – 0.332; lowest = 0.015; highest = 0.675). *Irregularity* for *E. coli* O157:H7 survival was significantly lower ($p<0.05$) in organically managed soils amended with manure (0.238 ± 0.035) than in conventionally managed soils amended with manure (0.350 ± 0.046).Sandy soil-manure mixtures (0.242 ± 0.043) showed a significantly ($p<0.05$) more stable survival of *E. coli* O157:H7 (less irregular) than loam soil-manure mixtures (0.346 ± 0.04) (Fig. 4).

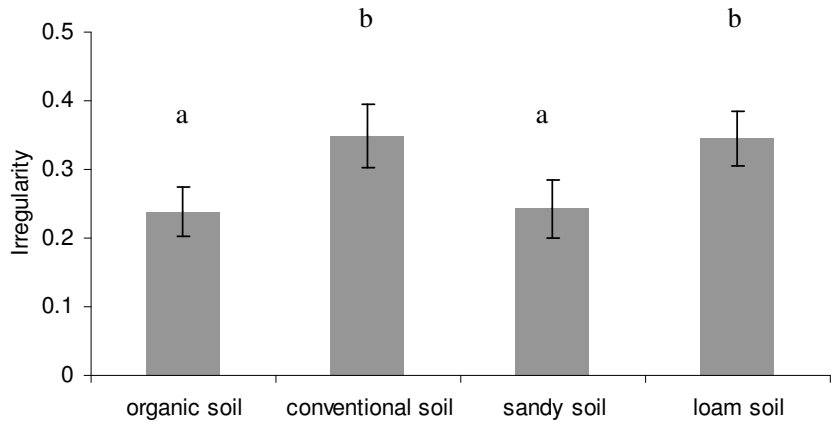

Fig. 4. Irregularity (ApEn) of declining *E. coli* O157:H7 populations in soil amended with manure by management type and soil texture. Vertical bars represent standard errors.

Relation between irregularity and characteristics of soil amended with manure. The *irregularity* of *E. coli* O157:H7 survival for all 18 pairs of soils amended with manure was positively correlated with the ratio between copiotrophic and oligotrophic bacteria (*copio/oligo*) ($r=0.52$, $p<0.01$) and *pH* ($r=0.46$, $p<0.01$), and negatively correlated with ammonium content (NH_4) ($r=-0.44$, $p<0.01$) and the fraction of sand particles (*sand*) ($r=-0.44$, $p<0.01$) (Fig. 5). Conventionally managed soils had the same set and direction of correlations but with lower levels of significance ($p<0.05$). *Irregularity* in organically managed soils was positively correlated with *copio/oligo* ($p<0.05$). Within sandy soils the *irregularity* showed a positive correlation with

copio/oligo (p<0.01) and negative with NH_4 (p<0.05). No significant correlations were found for loamy soils (Table 1).

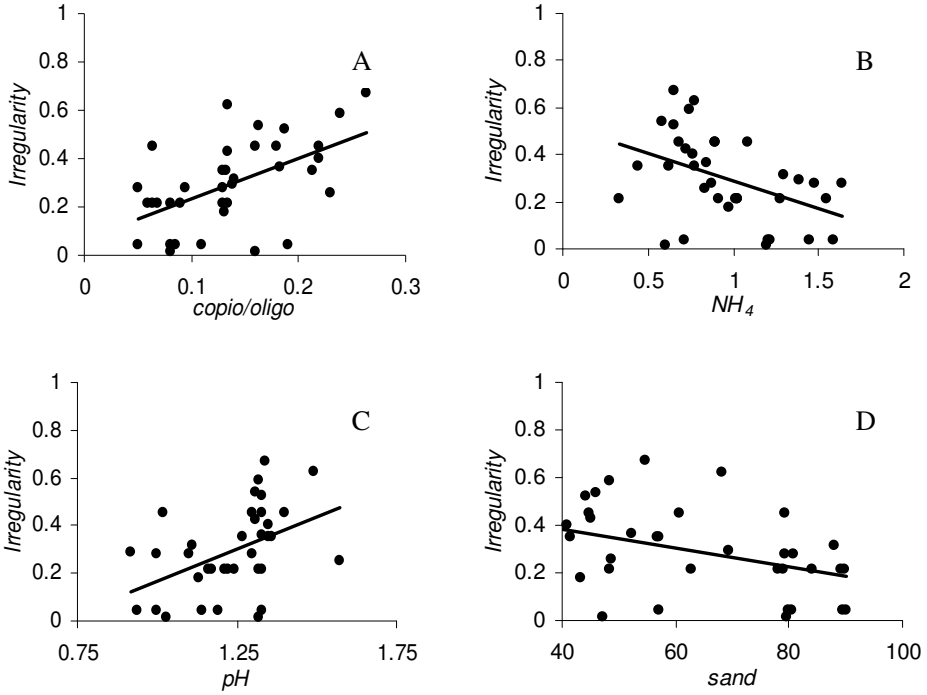

Fig. 5. Scatter plots and regression lines between the irregularity of declining *E. coli* O157:H7 populations and A) ratio of copiotrophic to oligotrophic bacteria, *copio/oligo*, B) NH_4, C) adjusted *pH* (see Materials and Methods), and D) sand content (*sand*) in all 36 soils amended with manure.

Step-wise multiple regression was performed to identify factors that can explain the observed variation in *irregularity* of *E. coli* O157:H7 survival (Table 2) which was normally distributed (Shapiro-Wilk test: p=0.15). *Copio/oligo* was the best predictor (p<0.05) of *irregularity* for all 36 soils as well as for organically (p<0.05) or conventionally (p<0.1) managed soils and for sandy soils (p<0.05). In all these cases increasing *copio/oligo* values resulted in significantly higher *irregularity*. Remaining variation in sandy soils was best explained by *C/N* (negative relation, p<0.05). No significant factors were found for loamy soils. Moreover, no significant correlation was found between *irregularity* and the time needed to reach the detection limit (Franz *et al.*, 2008) for *E. coli* O157:H7 survival.

Table 1. Pearson correlations between *irregularity* of *E. coli* O157:H7 survival and manure-amended soil characteristics.

	copio/oligo	*NH_4*	*Sand*	*pH*
Overall	0.52**	-0.44**	-0.44**	0.46**
Sandy soils	0.61**	-0.48*		
Loamy soils				
Organic soils	0.54*			
Conventional soils	0.53*	-0.49*	-0.52*	0.49*

No value: no significant relation

* significant at the 0.05 level (2-tailed)

** significant at the 0.01 level (2-tailed)

Table 2. Best regression models for *irregularity* of *E. coli* O157:H7 survival in 36 soils amended with manure

Group	Model	p-value	R^2
Overall	$1.62^{***}(\pm 0.45) \times$ ***copio/oligo***	0.001	0.27
Sandy soils	$1.15^{***}(\pm 0.33) + 1.87^{***}(\pm 0.59) \times$ ***copio/oligo*** $- 0.08^{***}(\pm 0.02) \times$ ***C/N***	0.006	0.49
Loamy soils	No model found	-	-
Organic soils	$1.49^{***}(\pm 0.58) \times$ ***copio/oligo***	0.02	0.29
Conventional soils	$1.66^{**}(\pm 0.66) \times$ ***copio/oligo***	0.023	0.28

* $p<0.15$, ** $p<0.10$, *** $p<0.05$

Influence of the manure-to-soil ratio on irregularity in *E. coli* O157:H7 survival. The different manure application rates (5%, 10% and 20%) showed only a minor influence on the irregularity of *E. coli* O157:H7 survival in manure-amended soils. The application rates had an effect only for conventionally managed soils: the *irregularity* negatively correlated (r=-0.58; $p<0.05$) with manure application rate (*rate*). Multivariate analysis of variance revealed that the application rate of manure in conventionally managed soils negatively influenced *irregularity*, according to the following equation: *irregularity* (model $r^2 = 0.34$; $p<0.05$) = 0.57 ($p<0.001$) – $1.2 \times 10^{-2} \times$ *rate* ($p<0.05$).

Discussion

The stability (in terms of the ability to return to an equilibrium) of autochthonous microbial communities after perturbation has been studied quite frequently (Ellis et al., 2003; Burauel and Baßmann, 2005; Girvan et al., 2005; Tobor-Kaplon et al., 2006), but the stability of introduced populations in natural substrates

was still unclear. In this study, we defined the stability of an introduced population as intensity of irregular dynamic changes in the population over time. In other words, the more stable a population is, the lower the number of changes in this population caused by unknown temporal processes. For the first time, we assessed the stability of *E. coli* O157:H7 survival and thereby indirectly the stability of the soils themselves, using Approximate Entropy (ApEn). ApEn was known to be a suitable method to characterize irregularity of changes in time series (Pincus, 1991). While ApEn has been used intensively in economy and medicine (Richman and Moorman, 2000; Pincus and Kalman, 2004), it has to the best of our knowledge not been applied in environmental microbiology. One data set of plant gas exchange values was analyzed by ApEn (Souza *et al.*, 2004; Souza *et al.*, 2005). The best results of an irregularity assessment for a time series can be achieved with a large number of data points. However, it has been proven mathematically that ApEn is also applicable for sequences as short as $N = 5$ points (Pincus and Singer, 1996). In our experiment, all *E. coli* O157:H7 survival curves have $N = 9$ points. Better estimates of regular temporal patterns and residual variability may be obtained by more frequent sampling (Zelenev *et al.*, 2005). Nevertheless, 9 data points proved to be sufficient to obtain interesting differences in ApEn between soil and management types and relationships of ApEn values with soil characteristics.

Previous survival studies with *E. coli* O157:H7 have been based on fitting pathogen populations over time to different nonlinear models, followed by comparison of calculated parameter values for different treatments (Franz *et al.*, 2008). The modified logistic model proved to be a suitable model for decline of *E. coli* O157:H7 in manure (Franz *et al.*, 2005). However, the intensity and predictability of variation around survival curves of enteropathogens can be an important issue for a risk assessment. ApEn analysis of declining *E. coli* O157:H7 populations in organically and conventionally managed soils amended with manure showed that the behavior of *E. coli* O157:H7 was significantly less irregular and thus better predictable in organic than in conventional soils. Although the average decline rates were similar for different management types (Franz *et al.*, 2008), the ApEn parameter varied for the same soils. So, ApEn analysis may be a sensitive method to differentiate between differently managed soils. The estimated temporal instability of enteropathogen populations can be used together with the classical way of decline rate calculation to increase the accuracy of estimated survival time periods of *E. coli* O157:H7 in soil amended with manure.

In this study, survival of *E. coli* O157:H7 was less irregular in sandy than in loamy manure-amended soils. In general loamy soils are characterized by a more heterogeneous structure (van Veen *et al.*, 1997). Soils amended with manure can be even more heterogeneous and dynamic systems. Spatial variation in soil nutrients, water content (Young *et al.*, 2001) and microbial communities (Mummey and Stahl,

2003) can lead to spatial variation in survival of introduced bacteria. To avoid such an influence of spatial variation, sampling errors (at the same sampling time) were subtracted from the residuals. Despite the correction for spatial heterogeneity in this study, higher temporal variation and irregularity in *E. coli* O157:H7 survival might still be the result of the presence of various micro-zones and protective pore spaces in the manure-amended loamy soils (van Veen *et al.*, 1997).

The best predictor for irregularity of *E. coli* O157:H7 survival in manure-amended soils was the ratio between copiotrophic and oligotrophic bacteria (*copio/oligo*). This means that the higher the density of oligotrophs compared to copiotrophs, the less irregular (more predictable) the survival of *E. coli* O157:H7 is. Oligotrophs are better able to live in nutrient-poor environments and are characterized by relatively slow growth rates, while copiotrophs can grow and reproduce better in rich environments, using a resource rapidly when available (Semenov, 1991). There are no truly constant environments, even in chemostat and turbidostat cultures. All organisms experience (and induce) dynamics in the nutritional state of their environment (Koch, 2001). However, temporal changes in oligotrophic populations and their influence on biological/chemical soil characteristics are less extreme and pronounced than those of copiotrophic populations (Zelenev *et al.*, 2006). Oligotrophication of ecosystems (e.g. by removal of easy available nutrient sources or enhancing stable organic matter content) could possibly increase the predictability of microbial community dynamics as well as that of enteropathogen survival.

Soils with low levels of *copio/oligo* frequently have a higher microbial diversity (van Bruggen and Semenov, 2000). Microbial diversity and species richness frequently are higher in organically than in conventionally managed soils (Mader *et al.*, 2002; van Diepeningen *et al.*, 2006), but in our study the difference was not large enough to be significant at $p<0.05$. The addition of manure as a carrier of *E. coli* O157:H7 might have masked the initial differences in soils characteristics. In addition, soils with higher microbial diversity are more resistant to stress and disturbance and consequently less susceptible to invasion (Girvan *et al.*, 2005). Maximal biodiversity in soils is generally reached under oligotrophic conditions with respect to available carbon sources, but mesotrophic conditions in terms of total organic carbon (van Bruggen and Semenov, 2000). Therefore, we assume that a high diversity of the microbial community together with minimal artificial disturbances (such as application of mineral fertilizers and pesticides or deep tillage) can lead to more equilibrated conditions in soil. Chemically and biologically balanced soils will prevent rapid and unpredictable changes in microbial populations and processes (van Diepeningen *et al.*, 2006).

It was previously observed that an ecosystem depending on more species in food chains of a longer length could be less stable (May, 1988). Nevertheless, there seems to be consensus that a minimum number of species is essential for ecosystem

functioning. A larger number of species is probably necessary for maintaining the stability of an ecosystem with constantly changing environments (De Ruiter *et al.*, 1995; Loreau, 2001; Botton *et al.*, 2006).The evident example of a system with changing environments is cultivated soil. Regular changes in the soil characteristics (application of fertilizers, crop harvesting, ploughing) can lead to irregular changes in microbial community structure (Botton *et al.*, 2006). This situation may also allow new species to successfully survive in a system with frequent changes in competing species. Therefore, soils that are under constant intensive pressure by farming procedures, more likely display unpredictable behavior of microbial communities and a lower predictability of enteropathogen survival.

One of the measurements of ecosystem stability is the magnitude of disturbance that can be absorbed before the system changes its structure by changing processes that control behavior (Botton *et al.*, 2006). This theory was corroborated in our research as well. With higher rates of manure application, organically managed soils did not change in irregularity of *E. coli* O157:H7 survival, while manure amendment significantly changed the irregularity for survival of *E. coli* O157:H7 in conventionally managed soils.

This is, to our knowledge, the first report where the stability of an introduced enteropathogen population in soil amended with manure was assessed. We propose that microbial communities in organically managed soils have more predictable behavior and are more stable thanks to a relatively high microbial diversity and oligotrophic condition with respect to easily available substrate. Organic soils showed a higher resilience to incremental rates of manure application. The results obtained in this research can be directly used in the risk assessment of *E. coli* O157:H7 survival in manure-amended soils as well as for characterization of soil health. Future research might focus on assessment of the stability of autochthonous populations and more frequent sampling, which may result in even more precise estimation of stability of the system under study.

Acknowledgments

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Chapter 6

Percolation and survival of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry

Semenov, A.V., van Overbeek, L., and van Bruggen, A.H.C. (2008) Percolation and survival of *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry.
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Abstract

The effect of cattle manure and slurry application on percolation and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was investigated for different soil depths after addition of water. Four treatments were chosen for the first set of experiments: 1) addition of inoculated farm-yard manure on the soil surface; 2) mixing of inoculated farm-yard manure with the top 10 cm of soil; 3) addition of inoculated slurry on the soil surface and 4) injection of inoculated slurry into the top 10 cm of the soil. Homogeneity of water distribution in the soil profile was confirmed by a non-destructive NMR method. Samples were collected at 10, 20, 30 and 40 cm depth after 1, 3, 5, 7 and 21 days. Survival data were fitted to a modified logistic model, and estimated survival times were compared. In the second set of experiments, pathogen-inoculated farm-yard manure was applied on the surface and inoculated slurry was injected into soil columns with one-month old lettuce plants. Bulk soil samples were collected after 1, 3 and 5 days as in the first set of experiments, and paired rhizosphere and bulk soil samples were collected along the total root length after 7 days. More pathogen cells percolated to greater depths after slurry than after manure application. The average survival time was generally shorter after surface application of manure or slurry than after incorporation into soil. Survival of *E. coli* O157:H7 was significantly longer in soil with slurry (29.7 ± 1.8 days) than in that with manure (17.8 ± 5.8 days), while survival of *Salmonella* serovar Typhimurium was equally high with manure and slurry, but the highest when injected into soil (43.4 ± 5.4 days). In soil with lettuce plants, the pathogens did not move beyond the rooting depth after one simulated rainfall event. The densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were not different in the rhizosphere compared to the bulk soil with manure, while the densities of these pathogens were higher by 0.88 ± 0.11 and 0.71 ± 0.23 log CFU gdw^{-1}, respectively, in the rhizosphere than in bulk soil with slurry. Our results suggest that surface application of manure may decrease the risk of contamination of ground water and lettuce roots compared to injection of slurry.

Introduction

In the last ten years food borne disease outbreaks have increasingly been associated with the consumption of fresh vegetables and fruits contaminated with human pathogenic bacteria (Beuchat, 1996; Sivapalasingam et al., 2004). A significant part of the outbreaks was attributed to *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Bovine manure and slurry are main environmental sources of these pathogens with average concentrations between 10^3 and 10^4 CFU gdw^{-1} of manure or slurry (Nicholson et al., 2005), but the density can be as high as 10^7 CFU gdw^{-1} of manure (Fukushima and Seki, 2004).

Utilization of organic manures such as farm-yard manure and slurry is the most economic and practical option for improving soil quality while providing an additional source of nutrients for growing plants as well. This is especially true for organic farms, where synthetic fertilizers cannot be used. Both organic and conventional soils can be fertilized with liquid slurry and/or farm-yard manure. However, farm-yard manure is more frequently used at organic farms.

The survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium is thought to be better in slurry than in farm-yard manure (Nicholson et al., 2005; Semenov et al., 2008b), but is also dependent on the way manure or slurry is applied to agricultural fields (Nicholson et al., 2005). Survival of the pathogens may range from several days (turned composted manure) till more than a year (non-aerated manure) (Kudva et al., 1998; Fremaux et al., 2007; van Elsas et al., 2007). This wide difference in survival time is caused by various abiotic factors such as temperature (Wang et al., 2004; Semenov et al., 2007), presence of oxygen (Semenov et al., 2008b) and chemical composition (Franz et al., 2008), as well as by biological factors (e.g. microbial community composition) (Jiang et al., 2002; Franz et al., 2007a; Semenov et al., 2007). The presence of plant roots is often neglected in controlled experiments, although root exudates may support survival of human pathogens by providing a supply of easy available nutrients (Klerks et al., 2007). Moreover, it has been shown that *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may become associated with the surface of plants growing in soil amended with contaminated manure (Natvig et al., 2002; Islam et al., 2004) and even be internalized into the plants (Solomon et al., 2002; Kutter et al., 2006; Franz et al., 2007; Klerks et al., 2007).

When microorganisms are introduced on or in soil, their movement is mainly determined by the flow of percolating water (Hekman et al., 1994). Water flow and the ultimate distribution of bacteria in soil is affected by soil texture, pH, temperature and the structure of root system in soil (Kemp et al., 1992). Like other bacteria, *E. coli* O157:H7 and *Salmonella* serovar Typhimurium are able to move through the soil profile with water after rainfall or irrigation and can even reach the ground water (Artz et al., 2005; Lang and Smith, 2007). In field experiments, 20% of *E. coli* applied with contaminated slurry to the field was found in drain water (Vinten et al., 2002). This

water can contaminate plants when it is used for irrigation. Since, *E. coli* O157:H7 can survive in well water up to 65 days (Artz and Killham, 2002), it is a high risk that private water supplies could be contaminated with enteric pathogens.

Laboratory transport studies can only mimic bacterial transport in field conditions to a certain extent. The natural heterogeneity in field soil leads to appearance of cracks and macropores through which water flow may occur, while relatively homogeneous soil is commonly used in laboratory experiments. This may underestimate movement of enteropathogens through the homogenized and possibly compacted soil. On the other hand, the presence of artificial boundaries (the so called "wall effect") and unexpected cracks may overestimate movement of water and bacteria through the soil in mesocosms. The wall effect can be minimized by inserting sand paper against the inner wall of soil columns, while cracks can be minimized by careful packing of the soil. Nuclear Magnetic Resonance (NMR) can be used to check the homogeneity of water distribution in a soil column. NMR is a non-destructive and non-invasive spectroscopic method to measure static and dynamic water behavior in heterogeneous substrates (van As and van Dusschoten, 1997). The data received from magnetic resonance images can give information about the spin density and spin relaxation values reflecting the interaction of water with the soil. These measurements have been proven to be highly correlated with water content in soils (van As and van Dusschoten, 1997).

While it was shown that water is the most important dispersal factor for percolation of bacteria in different types of soil (van Elsas et al., 1991; Hekman et al., 1994) as well as for percolation of enteropathogens under various management practices (Gagliardi and Karns, 2000), the movement and distribution of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil after application of manure and slurry is still unclear. It is also not clear if and how survival of enteric pathogens is influenced by the depth of the soil where they end up after transport through the soil.

The objectives of our study were: 1) to determine the extent of percolation of water and *E. coli* O157:H7 and *Salmonella* serovar Typhimurium from contaminated manure or slurry through a soil column; 2) to determine the influence of application methods of manure and slurry on percolation and survival of these pathogens at different depths in a soil column; 3) to determine the influence of plant roots on percolation and survival of the pathogens, applied with manure or slurry, at different depths in bulk soil and the rhizosphere.

Materials and methods

Bacteria. *E. coli* O157:H7 strain B6-914 *gfp*-91 was provided by Fratamico (Fratamico et al., 1997). The strain had been modified from strain SEA 13B88 (from the outbreak linked to Odwalla apple cider; Food and Drug Administration, 1996), so that it contained green fluorescent protein (*gfp*) (pGFO cDNA vector) and ampicillin

resistance, while the Shiga-like toxins ($Stx1^-$ and $Stx2^-$) were deleted. These changes did not result in significant differences in survival in nutrient media compared to the wild-type strain (Fratamico et al., 1997). *Salmonella* serovar Typhimurium MAE 119 (ΔagfD101 *saw*) was obtained from Römling (Römling et al., 1998; Römling et al., 2000). This strain carried resistance to kanamycin and gentamycin and carried the *gfp* gene after transformation with the PAG408 mini-transposon. No differences between the wild-type of *Salmonella* serovar Typhimurium and its transformed form were found (Römling et al., 2000). Green fluorescence of both *gfp*-transformed strains was checked under UV light. Stock cultures were stored in 30% (w/w) glycerol at -80°C.

Farm-yard manure and slurry. Slurry and fresh manure without urine from organically managed Frisian Holstein cows on a standard 50% grass/clover-silage + 50% dried grass diet were used for the experiments. Manure was mixed with straw (90% manure and 10% straw (kg/kg, dry weight) and stored for nearly one month in a heap (sampling depth 20cm, 30-40 °C at that depth) at the organic farm of L.M.M. Pool (Bennekom, The Netherlands). About 5 kg of manure from this heap as well as 5 litres of slurry from the farm reservoir were collected in June 2006 for the first set of the experiments and in June 2007 for the second set, homogenized and stored in closed plastic bags at 5 °C for two weeks before the start of the experiments, which were carried out in a time span of 10 months. Chemical characteristics of farm-yard manure and slurry are presented in Table 1.

Table 1. Chemical characteristics of manure and slurry used in both sets of the experiments

Substrate	Variable						
	Dry matter content, %	pH	Organic matter, g kg^{-1}	$N\text{-}NO_3$, g kg^{-1}	$N\text{-}NH_4$, g kg^{-1}	DON, g kg^{-1}	DOC, g kg^{-1}
Manure	35.2	9.1	74.75	0.00	1.00	0.17	5.02
Slurry	5.5	8.5	60.93	0.00	0.60	0.37	12.48

Soil. Soil was collected from a field which had not been cultivated over the last 3 years and was covered with grass at the organic experimental farm Droevendaal (Wageningen University and Research center, the Netherlands). Throughout the sampled field, 10 soil sub-samples (20 cm deep) were collected between the plants and mixed in June 2006 for the first set of the experiments and in June 2007 for the second set. All samples were transported to the laboratory in plastic bags, thoroughly mixed, sieved through 0.5 cm mesh to remove plant parts and earthworms and stored at 5 °C until the start of the experiments.

Tube preparation. Fifteen (for the first set of the experiments) and eighteen (for the second set) PVC-U grey tubes were prepared. The dimensions of each tube were: length 50 cm and 5 cm diameter. Their bottom part was closed with a cap of the same material and diameter. To prevent a "wall effect" the internal surface of the tubes was covered with sand paper. Four holes of 1 cm diameter each and with the same distance from each other were drilled around the tubes at 13, 23, 33 and 43 cm height from the top (16 holes per tube in total). The cylinders were also cut in half vertically, from the bottom to the top. That was done to be able to take samples from the rhizosphere and bulk soil at the end of the second set of experiments. All openings were closed with waterproof power tape at the start of each experiment. The tubes were filled with the sieved soil from the bottom to 3cm from the top. The top 3 cm were left to add manure and slurry at the start of the experiments. The tubes were placed in a box vertically and were left undisturbed for a week to allow settling of the soil before manure or slurry was applied. The bulk density was 1.3 g cm^{-3}.

Inoculation of manure and slurry. Bacterial inocula were prepared in Erlenmeyer flasks containing 150 ml fresh two-times diluted LB broth with 50 μg ml^{-1} ampicillin (Sigma-Aldrich Chemie Gmbh, Germany) for *E. coli* O157:H7 and with kanamycin (50 μg ml^{-1}) for *Salmonella* serovar Typhimurium and were incubated at 37 °C on an orbital shaker (200 rev min^{-1}) for 18 h. Liquid cultures were centrifuged at 10,000 × g for 10 min, washed three times and resuspended in sterile distilled water. The number of cells ml^{-1} of suspension was determined using the spectrophotometer, where OD 0.7 at 630 nm in a 1 ml cuvet was equal to 1×10^9 CFU ml^{-1}. Prepared inocula were added with a pipette to manure or slurry and mixed thoroughly within a double layer of plastic autoclavable bags for 5 min.

Setup of the experiments. Two series of experiments were carried out to investigate percolation and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium at different depths in the soil profile either in the presence or absence of plants. For the first set of the experiments, separate experiments were carried out with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, while for the second set, both pathogens were used simultaneously.

Four treatments were chosen for the first set of experiments: 1) addition of inoculated farm-yard manure on the soil surface; 2) mixing of inoculated farm-yard manure with the top 10 cm of the soil; 3) addition of inoculated slurry on the soil surface and 4) injection (by syringe) of inoculated slurry into the top 10 cm of the soil.

In the second set of experiments, a week after the tubes were filled with soil, one seed of iceberg lettuce (*Lactuca sativa* L. cv. Tamburo) was sown per tube. Farm-yard manure or slurry was applied to the soil tubes with 1 month old lettuce plants. Two treatments were compared: 1) addition of inoculated farm-yard manure on the soil

surface (manure was located at least 0.5 – 1 cm distance from the lettuce stem); and 2) injection (by syringe) of inoculated slurry into the top 10 cm of the soil (at the same distance from the lettuce stem).

To have the same dry weight of amendments as well as the same concentration of manure and slurry (10% kg kg^{-1}) for each treatment, 15 g of inoculated manure or 75 ml of inoculated slurry were added to the tubes with soil according to the treatments outlined above. Moreover, to equalize the amount of water added to the tubes as well as to mimic the influence of rain fall on percolation of the pathogens, 90 and 30 ml of water were added to the soil with manure and slurry, respectively, using an infusion pump (Harvard apparatus, USA) with a flow of 2.2 ml min^{-1}.

Control tubes for chemical measurements were prepared with non-inoculated manure or slurry. All tubes were transported to the Unifarm (Wageningen University and Research Center, The Netherlands) greenhouse maintained at 16 °C, with 19 hours of supplemental light and a relative humidity of 50%. Each treatment had 2 replicates in each experiment. For the first set of experiments, each experiment was repeated three times, and for the second set two times.

Sampling procedure. Samples (approx. 0.5 g) were collected at 10, 20, 30 and 40 cm depth after 1, 3, 5 days (and additionally after 7 and 21 days for the first set of experiments) and at a distance of 2, 4, 6 and 8 cm from the root tip 7 days after manure or slurry application (only for the second set of experiments). During this last sampling time, both rhizosphere and bulk soil samples were collected at the same depths. The rhizosphere samples weighted on average 0.4 g and the bulk soil samples 0.5 g.

All samples were put in pre-weighed dilution tubes with 4.5 ml of 0.1% peptone buffer and weighed. Samples were vortexed and sonicated for 30 s (Branson 5200, 120-W output power, 47 kHz). Ten-fold dilution series were prepared with sterile distilled water, and 50 µl of the two highest dilutions per sample were plated in duplicate on sorbitol-MacConkey agar (Oxoid) with ampicillin (50 µg ml^{-1}) for *E. coli* O157:H7 and on Luria-Bertani agar with kanamycin (50 µg ml^{-1}) for enumeration of *Salmonella* serovar Typhimurium, respectively. After adding approximately 20 sterile glass beads per Petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass beads to spread the inoculum over the surface of the plate. Fluorescent bacterial colonies were counted under a UV lamp (365 nm UV-A, PL-S, Philips, Eindhoven, the Netherlands) after incubation at 37 °C for 24 h. Fluorescent colonies made up 95-99% of all colonies on a plate. Fluorescent colony-forming units (CFU) were calculated per gram of dry soil. The calculated detection limit was 100 CFU gdw^{-1}.

NMR. A nuclear magnetic resonance (NMR) instrument that can measure ^{1}H was used to determine the movement and distribution of water in a non-destructive and non-

invasive way in soil columns with non-inoculated manure and slurry on the surface or incorporated in the top 10 cm. The NMR system consists of an Avance console (Bruker, Karlsruhe, Germany) and a superconducting magnet with a 50 cm vertical free bore (Magnex, Oxford, United Kingdom), which generates a magnetic field of 3 T (128 MHz proton frequency). A radio frequency coil was used for detection of the signal. The vertical bore of the magnet allows the measurement of soil tubes in vertical direction. A remote climate control unit was used to maintain optimal conditions. For the measurements a pulsed field gradient – stimulated echo – multi spin echo (PFG-STE-MSE) was used. The data received from the magnetic resonance images gave information about the spin density and spin relaxation values (T_1 and T_2) reflecting the interaction of water with the soil. T_2 measurements are highly correlated with the water content of the soil (van As and van Dusschoten, 1997). T_2 relocation time was determined for every cm of the soil columns, since the echo train was adjusted for every PFG step. The NMR signal intensity of an additional tube with water was used for a calibration. Fifty slices (every 1cm) were measured per soil tube. A technical discussion of mathematical and physical properties of the NMR instrument can be found elsewhere (van As and van Dusschoten, 1997; Homan et al., 2007; van As, 2007).

Chemical characterization. Fresh samples of soil at 10, 20, 30 and 40 cm depth after manure and slurry application from the control pots were analyzed at the start and at the end of the experiments. The pH was measured in water suspension 1 : 2.5 (g/v) with an Inolab Level 1 pH-meter (WTW GmbH, Weilheim, Germany). Nitrate (NO_3^-), ammonium (NH_4^+) and total dissolved nitrogen (N_{ts}) content were determined colorimetrically in a solution of 0.01 M $CaCl_2$ with an Autoanalyzer II (Technicon Instrument Corporation, Tarrytown, New York). Dissolved organic nitrogen (DON) was calculated as the difference between N_{ts} and the amount of nitrogen present as NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) was measured by a carbon analyzer in a soil extract of 0.01 M $CaCl_2$. Dried (24 h at 40 °C) manure and slurry samples were ground and used to measure total carbon (C_{total}) and total nitrogen (N_{total}) by the Dumas method followed by detection by a Fisons element analyzer type EA 1108 (Therom Finnigan Italia S.P.A., Milan, Italy). Water content was measured by comparison of fresh and dried (40 °C for 24 h) weight of samples.

Statistical analysis. The number of fluorescent colonies per Petri plate was expressed as CFU gdw^{-1} and standard deviations were calculated for every treatment, time and depth combination. To describe the decline in CFUs over time (for Exp. 1), log-transformed data were fitted (separately for each replication) to a modified logistic function by nonlinear regression (Gauss-Newton method): $C_t = a / (1 + c \times e^{(-m \times t)})$, where C_t is the log CFU gdw^{-1} at time t (days), a is the upper asymptote (CFU gdw^{-1}), c

is a parameter for the shoulder (days), and *m* is a slope parameter for the rate of change ($days^{-1}$) (SAS® system for Windows version 9.1, SAS Institute Inc, Cary, NC, USA, 2003). The upper asymptote was kept constant as 7 log CFU gdw^{-1}. This model was selected since the R^2 was the highest for all treatment × depth combinations compared to other tested models (linear, exponential, Weibull, logistic). Moreover, this model had shown excellent fits for decline data of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium during previous studies (Franz et al., 2005; Semenov et al., 2007). For the second set of experiments, decline rates were calculated by linear regression of CFU's over time. The performance of both non-linear and linear models was assessed by calculating the regression coefficient (R^2) and significance level. In addition to the model parameters, the time needed to reach the detection limit (2 log CFU gdw^{-1}) was calculated (*survival time* in days). The two-sided t-test was used to distinguish differences in estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (unpaired t-test) as well as in population density between rhizosphere and bulk soil (paired t-test). The influence of type of manure or slurry application, sampling depth on estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was assessed by General Linear Model (GLM) and Mixed Model (Mixed) procedures (SAS® system for Windows version 9.1, SAS Institute Inc, Cary, NC, USA, 2003) separately for each pathogen for the first set of experiments and for both pathogens together for the second set. Treatment (combination of manure type and application method) and soil depth were considered fixed effects, while repetition of the experiments and replicate within each experiment were considered as random effects in the Mixed Model. A split-plot design was used with depth as subplot and treatment as main plot factors. In the experiments with lettuce both pathogen and treatment were considered as a main plot factors and depth again as a subplot factor.

Stepwise multiple regressions were conducted by the REG procedure (SAS® system for Windows version 9.1) to determine to what extent chemical parameters could explain variation in survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. Regression analyses were conducted on normalized data to avoid possible nonlinear relations. The following parameters were included in the analysis: survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (days), organic matter (%), log $N\text{-}NO_3$ (mg kg^{-1}), $N\text{-}NH_4$ (mg kg^{-1}), DOC (mg kg^{-1}), DON (mg kg^{-1}) and log pH. Variables in the regression models were considered significant at the 0.1 level. Models were restricted to a maximum of two parameters. Multiple regressions were conducted for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium separately for the experiments without lettuce and together for the experiments with lettuce.

Results

NMR and soil water content. Visual analysis of NMR images of water intensity showed absence of soil cracks as well as absence of a wall effect for all treatments

(Fig. 1). Therefore, it was assumed that the distributions of soil and water in the tubes were generally homogeneous. Moreover, the water content determined after drying of soil samples at 40 °C was very similar to the water content determined by NMR (Fig. 2).

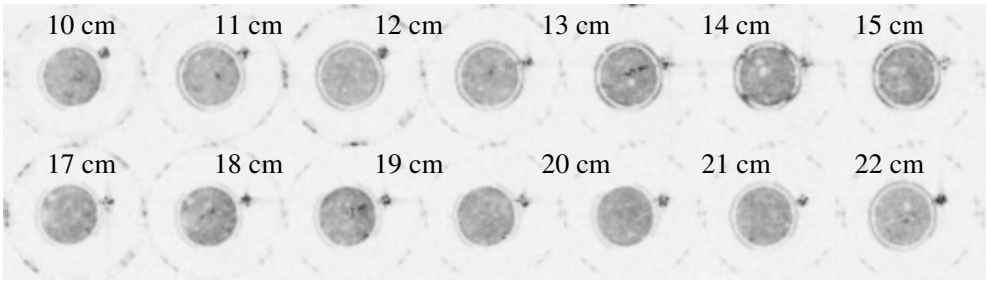

Fig 1. Representative examples of water distribution (intensity of grey color represents water content) at 10-23 cm depth in a soil tube with manure applied on top of the soil after 1 day for the first set of the experiments as measured by NMR

Chemical characteristics of the soil. In the first set of the experiments, concentrations of $N\text{-}NO_3$, DON and DOC significantly ($p<0.05$) increased with depth at the beginning as well as at the end of the experiment, while $N\text{-}NH_4$, pH and organic matter were equally distributed during 21 days of the experiments (Table 2). Incorporation of manure and injection of slurry lead to significantly higher concentrations of all four soluble compounds ($N\text{-}NO_3$, $N\text{-}NH_4$, DON and DOC) in upper layers of the soil tubes compared to treatments where manure and slurry were spread on the soil surface.

Comparison of chemical characteristics for slurry and manure treatments at the beginning of experiments in the second set (Table 3) showed significantly higher concentrations of $N\text{-}NO_3$ and DON at 10 cm depth after injection of slurry, while DOC was higher at 40 cm after application of manure compared to injection of slurry. At the end of the experiments, $N\text{-}NH_4$ was significantly higher in manure treatments for the first 30 cm and the concentration of $N\text{-}NO_3$ was lower compared to the slurry treatments.

Percolation of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns without plants. The initial calculated inoculum density of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was 7.8 and 8.1 log CFU gdw^{-1} of soil (for upper 10 cm), respectively, both for manure and slurry treatments. One day after application of manure (spread on the soil surface or mixed in the top 10cm of soil) and slurry (spread on the soil surface or injected into 10 cm of soil) followed by rain fall simulation, the pathogens and water were distributed throughout the soil tubes (Fig. 2). In general, the distribution of the pathogens for the different treatments was similar to

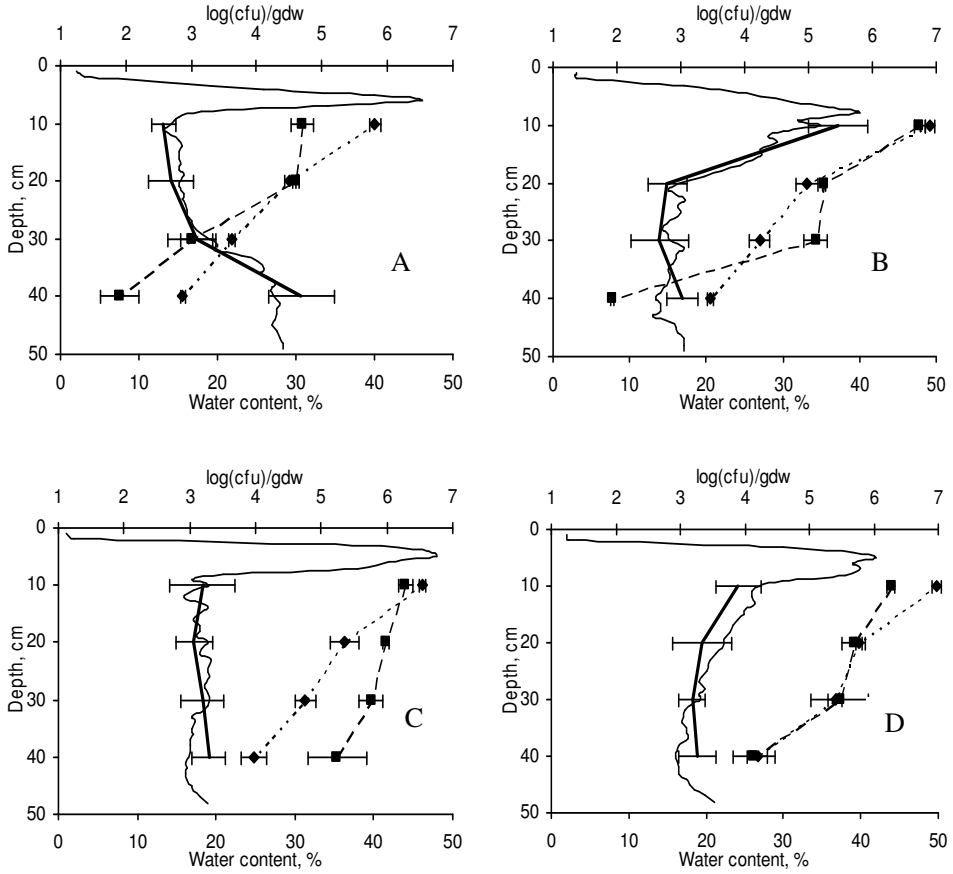

Fig. 2. Comparison of two methods for measurement of water content by the common procedure (bold solid line) and NMR (thin solid line) in a soil profile with A) manure spread on the soil surface B) manure mixed with the top 10cm of soil C) slurry spread on the soil surface D) slurry injected into 10cm of soil after 1 day of the experiment 1. Dashed lines represent density of *E. coli* O157:H7 (squares) and *Salmonella* serovar Typhimurium (diamonds) after 1 day for the first set of the experiments. Vertical bars represent standard errors.

each other (the highest density at 10 cm and the lowest at 40 cm). The most pronounced difference between the densities of the pathogens at the top (10 cm) and at the bottom (40 cm) of the soil columns was for treatments with applications of manure (on average, 4.1 and 3.3 log CFU gdw^{-1}, for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, respectively) which was significantly ($p<0.05$) higher than the

Table 2. Chemical characteristics of soil in tubes at 10, 20, 30 and 40 cm depth, 1 and 21 days after manure/slurry application in the first set of the experiments

Treatment	Variable[1]							
	$N\text{-}NO_3$, mg kg^{-1}		$N\text{-}NH_4$, mg kg^{-1}		DON, mg kg^{-1}		DOC, mg kg^{-1}	
Depth, cm	start[2]	end[3]	start[2]	end[3]	start[2]	end[3]	start[2]	end[3]
Soil used in Experiment 1	46.93		2.15		4.52		73.94	
manure spread on surface								
10	17.5[a]	6.3[a]	2.28[b]	2.17[b]	6.17[a]	6.54[a]	76.5[a]	73.2[b]
20	24.8[a]	7.5[a]	2.30[b]	2.21[c]	7.75[a]	7.88[c]	80.1[b]	77.8[a]
30	40.7[a]	34.4[b]	2.12[c]	2.08[b]	9.42[c]	8.26[b]	82.5[c]	77.2[a]
40	73.4[b]	81.2[a]	2.25[b]	2.23[a]	9.23[b]	9.72[c]	84.9[b]	83.5[a]
manure mixed with top 10cm								
10	24.4[b]	8.2[b]	2.39[c]	2.19[b]	8.99[d]	7.33[c]	78.9[b]	75.8[c]
20	30.6[b]	7.5[a]	2.30[b]	2.31[d]	8.12[b]	7.78[c]	78.2[a]	77.1[a]
30	43.3[a]	31.1[b]	2.21[d]	2.06[b]	8.64[b]	8.15[b]	81.9[c]	79.0[b]
40	75.9[b]	82.0[a]	2.26[b]	2.18[a]	9.12[b]	9.02[b]	85.7[c]	84.2[a]
slurry spread on surface								
10	18.5[a]	6.8[a]	2.11[a]	1.74[a]	7.01[b]	6.25[a]	75.7[a]	72.3[a]
20	22.0[a]	8.1[a]	1.88[a]	1.44[a]	7.88[a]	6.55[a]	80.2[b]	78.2[a]
30	47.6[b]	30.8[a]	1.97[b]	1.62[a]	8.59[b]	7.49[a]	78.3[a]	77.4[a]
40	67.8[a]	83.4[a]	2.20[b]	2.87[b]	9.13[b]	7.88[a]	84.5[b]	83.1[a]
slurry injected into 10cm of soil								
10	27.2[c]	9.0[b]	2.30[b]	2.10[b]	7.75[c]	6.71[b]	79.1[b]	72.9[b]
20	21.4[a]	7.5[a]	2.02[a]	1.59[b]	8.20[b]	6.94[b]	81.5[c]	77.5[a]
30	42.1[a]	28.9[a]	1.87[a]	1.96[b]	8.01[a]	7.37[a]	80.6[b]	77.1[a]
40	72.3[b]	84.1[b]	2.05[a]	2.28[a]	8.96[a]	7.97[a]	83.6[a]	84.9[b]

[1] pH and Organic matter are not shown since no significant differences were found among application methods and depths

[2] at the beginning of the experiment, 1 day after addition of water to the soil tubes

[3] at the end of the experiment after 21 days

[a, b] significant ($P < 0.05$) differences among manure types and application methods at the same depth for each variable

Table 3. Chemical characteristics of soil in tubes at 10, 20, 30 and 40 cm depth, 1 and 7 days after manure/slurry application in the second set of the experiments

Treatment	Variable[1]							
	$N\text{-}NO_3$, mg kg^{-1}		$N\text{-}NH_4$, mg kg^{-1}		DON, mg kg^{-1}		DOC, mg kg^{-1}	
Depth, cm	start[2]	end[3]	start[2]	end[3]	start[2]	end[3]	start[2]	end[3]
Soil used in Experiment 2	47.37		2.04		4.50		74.59	
manure spread on surface								
10	18.3 [a]	1.8 [a]	2.24 [a]	2.05 [b]	6.20 [a]	6.59 [a]	75.1 [a]	70.9 [a]
20	21.5 [a]	5.2 [b]	2.33 [b]	2.19 [b]	7.12 [a]	7.74 [b]	82.4 [a]	76.5 [a]
30	41.2 [a]	30.9 [a]	2.10 [b]	1.97 [b]	9.05 [b]	7.60 [a]	81.9 [a]	76.6 [a]
40	70.3 [a]	80.7 [a]	2.19 [a]	2.24 [a]	9.41 [a]	8.79 [a]	85.2 [b]	82.8 [a]
slurry injected into 10cm of soil								
10	25.9 [b]	4.9 [b]	2.25 [a]	1.89 [a]	7.91 [b]	6.04 [a]	76.6 [a]	71.7 [a]
20	20.1 [a]	4.9 [a]	2.08 [a]	1.24 [a]	8.24 [b]	6.34 [a]	82.4 [a]	77.7 [a]
30	42.5 [a]	27.8 [a]	1.80 [a]	1.49 [a]	7.81 [a]	6.92 [a]	80.5 [a]	77.1 [a]
40	74.2 [b]	83.2 [b]	2.12 [a]	1.98 [a]	9.24 [a]	7.58 [a]	81.8 [a]	84.8 [a]

[1] pH and Organic matter are not shown since no significant differences were found among application methods and depths

[2] at the beginning of the experiment, 1 day after addition of water to the soil tubes

[3] at the end of the experiment after 7 days

[a, b] significant ($P < 0.05$) differences between manure and slurry treatments at the same depth for each variable

difference in density of the pathogens between top and bottom after application of slurry (2.2 and 1.8 log CFU gdw^{-1}, for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, respectively).

Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil tubes. After the first day, *E. coli* O157:H7 and *Salmonella* serovar Typhimurium declined in all treatments and at all depths (10, 20, 30 and 40 cm) (Fig. 3 and 4). There were significant ($p<0.01$) interactions between manure/slurry treatment and soil depth ($p<0.01$). When manure was applied on the soil surface, *E. coli* O157:H7 was not detected after 7 days at 30 cm depth and already after 1 day at 40 cm depth. In the treatment with manure incorporated into the first 10 cm of soil, the density of *E. coli* O157:H7 was below the detection level after 1 day at 40 cm depth. In both manure

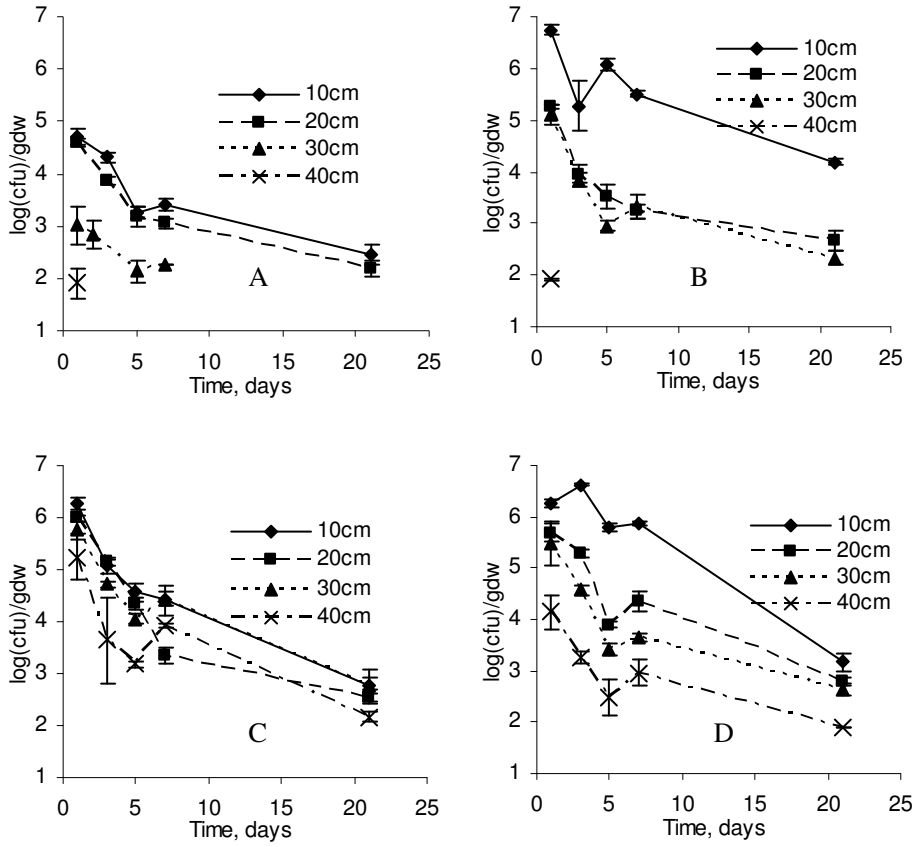

Fig. 3. Average density of *E. coli* O157:H7 during 21 days at 10, 20, 30 and 40 cm depth in a soil inoculated with A) manure spread on the soil surface B) manure mixed with the top 10cm of soil C) slurry spread on the soil surface and D) slurry injected into 10cm of soil. Vertical bars represent standard errors.

treatments, *Salmonella* serovar Typhimurium showed a similar poor survival at 40 cm depth after 1 and 3 days before the detection limit was reached.

In case of slurry, *E. coli* O157:H7 was detected both after surface application and injection at all depths during 21 days of the first set of experiments, while *Salmonella* serovar Typhimurium survived better at 10-30 cm depth than at 40 cm depth, where it survived for only for 5 - 7 days.

When log CFUs of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were regressed over time using the modified logistic survival model, there were significant fits ($p < 0.05$) for all treatments, depths and replicates with an average

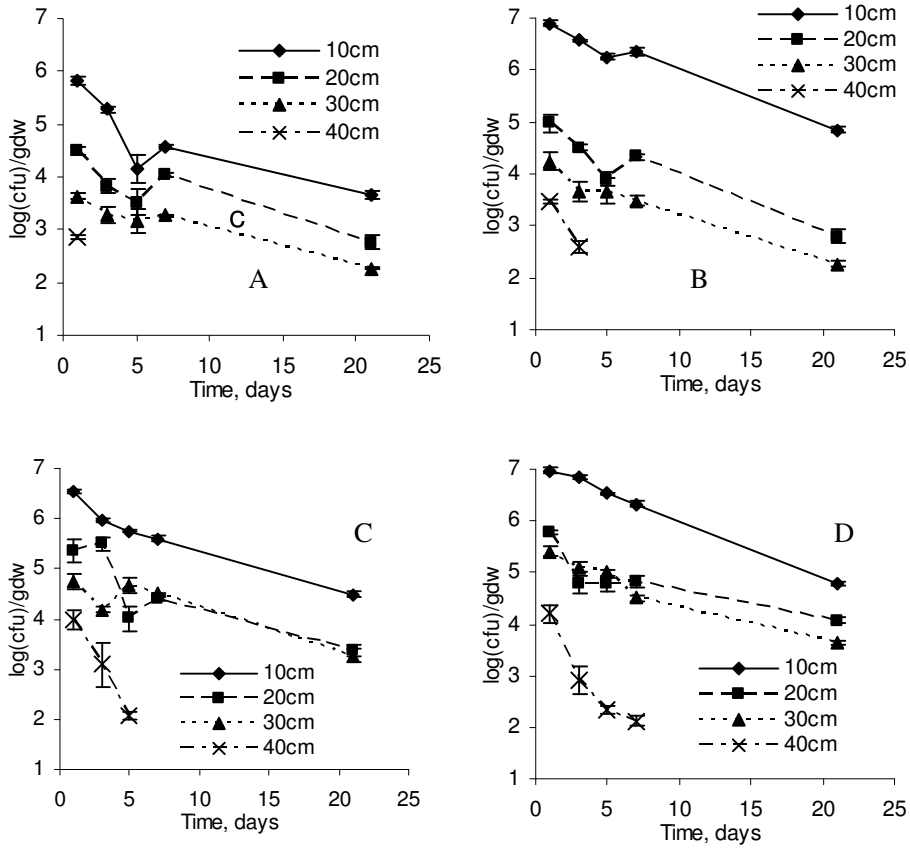

Fig. 4. Average density of *Salmonella* serovar Typhimurium during 21 days at 10, 20, 30 and 40 cm depth in a soil inoculated with A) manure spread on the soil surface B) manure mixed with the top 10cm of soil C) slurry spread on the soil surface and D) slurry injected into 10cm of soil. Vertical bars represent standard errors.

pseudo-R^2 of 0.92 ± 0.02 and 0.96 ± 0.03, respectively, for *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (Fig. 5). Estimated *survival time* of *Salmonella* serovar Typhimurium was significantly (unpaired test; $p < 0.05$) higher than *survival time* of *E. coli* O157:H7 at all depths except at 40 cm. Average survival time of *E. coli* O157:H7 at all depths was significantly higher after application of slurry (29.7 ± 1.8 days) than after manure application (17.8 ± 5.8 days). For *Salmonella* serovar Typhimurium, there was no overall difference between manure and slurry ($p>0.05$), but injection of slurry significantly increased survival (42.5 ± 5.5 days) compared to surface application of manure (25.7 ± 6.5 days). The longest survival time was

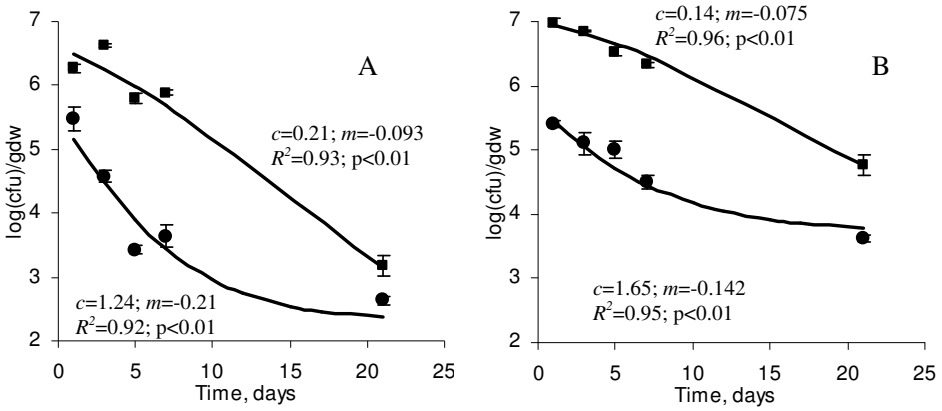

Fig. 5. Representative examples of fitting observed densities (closed squares and circles) of *E. coli* O157:H7 (A) and *Salmonella* serovar Typhimurium (B) with a modified logistic model (solid lines) at 10cm (squares) and at 30cm depth (circles) after injection of slurry into 10cm of soil. Vertical bars represent standard errors.

estimated for 10 cm depth when manure was incorporated into the top 10 cm of soil (50.1 ± 5.0 for *E. coli* O157:H7 and 65.7 ± 5.3 days for *Salmonella* serovar Typhimurium, respectively) (Fig. 6). The next longest survival time was estimated for slurry injected in soil (29.9 ± 1.8 days for *E. coli* O157:H7 and 42.5 ± 5.5 days for *Salmonella* serovar Typhimurium). The mixed model showed a significant effect ($p<0.01$) of the manure/slurry treatment, sampling depth as well as an effect of the interaction between treatment and sampling depth on estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium.

Step-wise multiple regression was performed to identify factors (Table 2) that can explain the observed variation in *survival times* of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium which were normally distributed (Shapiro-Wilk test: p=0.78 and p=0.95, respectively). According to the following equation: *survival time* = -270.33 (±99.37) [$p < 0.05$] + 129.75 (±43.81) × ($N\text{-}NH_4$) [$p < 0.05$, $R^2_{part}=0.69$], $N\text{-}NH_4$ was the best predictor for survival time of *E. coli* O157:H7 applied to soil with manure. Increasing concentrations of $N\text{-}NH_4$ resulted in significantly higher survival time of the pathogen. No significant factors were associated with survival time of *Salmonella* serovar Typhimurium.

Percolation of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns with lettuce plants. In the second set of experiments, manure and slurry were inoculated with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium at 6.6 and 7.1

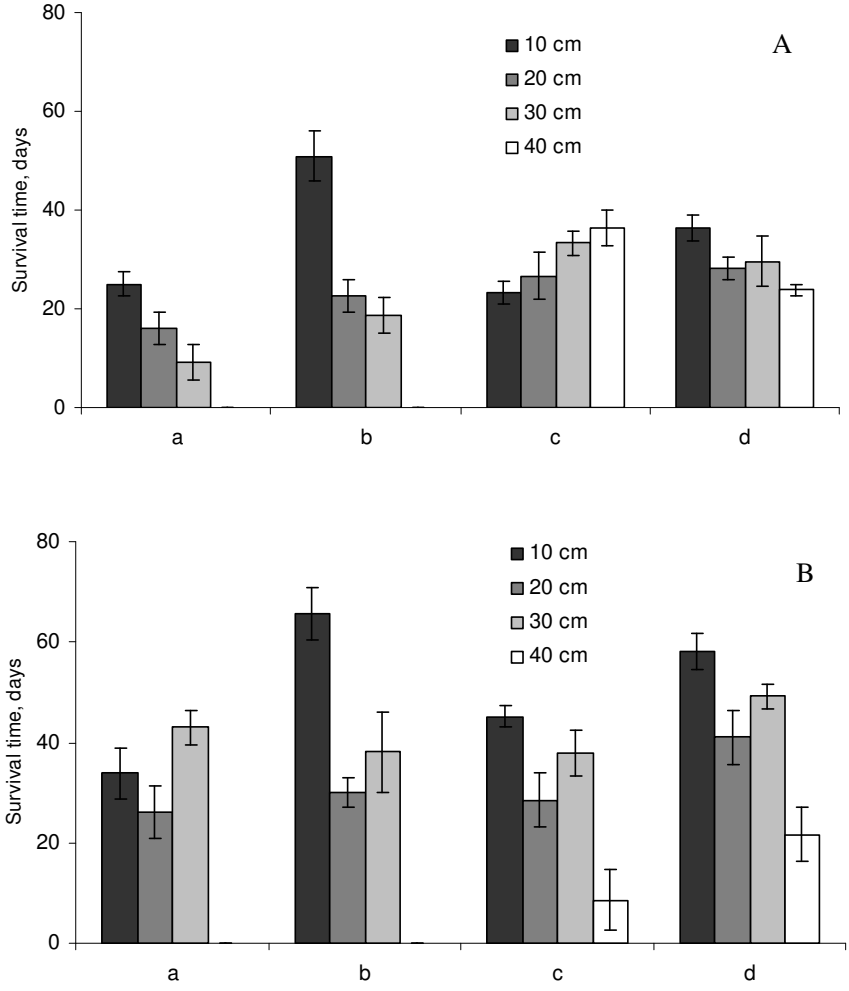

Fig. 6. Average estimated survival time by modified logistic model for A) *E. coli* O157:H7 and B) *Salmonella* serovar Typhimurium at 10, 20, 30 and 40 cm depth in a soil inoculated with a) manure spread on soil surface b) manure mixed with top 10cm of soil c) slurry spread on soil surface and d) slurry injected into 10cm of soil. Horizontal bars represent standard errors.

log CFU gdw^{-1} of soil, respectively, and applied to soil tubes with one month old lettuce plants. The densities of the pathogens after one day in the top 20 cm of the soil

columns were similar to those observed in the first set of the experiments without lettuce plants. However, neither of the pathogens were detected at 30 and 40 cm depth.

Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil tubes with lettuce plants. Survival of the pathogens was determined during the first week after application of manure or slurry (Table 4). Density of *E. coli* O157:H7 significantly decreased at 10 and 20 cm depth when manure was spread on the soil surface. A significant decline was also observed at 20 cm depth, when inoculated slurry was injected. Contrary to that, changes in density of *Salmonella* serovar Typhimurium were not significant, although they tended to be lower after 5 days of manure or slurry application compared to the first day. Samples at 30 and 40 cm depths remained negative for both pathogens for the duration of the experiments. Due to the limited number of samplings (N=3), instead of non-linear regression, linear regression was performed to check main trends in *E. coli* O157:H7 and *Salmonella* serovar Typhimurium survival (Table 4). No significant relations were found between decline rates and chemical soil characteristics. The mixed model showed a significant effect ($p<0.001$) of the manure/slurry treatment, sampling depth and pathogen, as well as the interactions between treatment, pathogen and sampling depth on the estimated survival time of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium (Table 4).

Seven days after inoculated manure and slurry were added to the soil tubes, bulk soil and soil from the rhizosphere of lettuce plants were collected at 2, 4, 6 and 8 cm from the root tip. When manure was spread on the soil surface no significant ($p>0.05$) differences in density of *E. coli* O157:H7 or *Salmonella* serovar Typhimurium were found between rhizosphere and bulk soil (Fig. 7). However, in treatments with injected slurry, densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were significantly higher (by 0.88 ± 0.11 and 0.71 ± 0.23 log CFU gdw^{-1}, respectively), in rhizosphere than in bulk soil.

Discussion

Similar to other studies on percolation and survival of enteropathogens in soil amended with manure or slurry (Gagliardi and Karns, 2000, 2002; Vinten et al., 2002; Artz et al., 2005; Lang and Smith, 2007), the distribution of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was affected by the type of the substrate which was added (manure or slurry); by the method of its application (e.g. spread on the soil surface or incorporating into the soil), as well as by the presence of lettuce roots. The survival of *E. coli* O157:H7 was on average 1.39 ± 0.12 times shorter than survival of *Salmonella* serovar Typhimurium for all treatments. A similar difference in survival for the pathogens was found in previous experiments (Franz et al., 2005; Semenov et al., 2007). For the first time, we showed that application of slurry led to higher density and

Table 4. Average survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium at 10, 20, 30 and 40 cm of soil profile with inoculated manure spread on soil surface and slurry injected into 10cm of soil in the second set of the experiments

Pathogen	Treat-ment	Depth cm	Log (cfu) gdw^{-1}			Decline rate, log(cfu) day^{-1}	Estimated survival time, days
			day 1	day 3	day 5		
E. coli O157:H7	Manure spread on surface	10	5.96^{a} (±0.32)	5.14^{b} (±0.28)	4.93^{b} (±0.13)	-0.26*	17.1
		20	5.06^{a} (±0.09)	4.30^{b} (±0.29)	4.39^{b} (±0.28)	-0.17*	20.9
		30	n/d	n/d	n/d	n/a	
		40	n/d	n/d	n/d	n/a	
	Slurry injected into 10cm	10	5.64^{a} (±0.31)	5.21^{a} (±0.54)	5.06^{a} (±0.36)	-0.15*	27.6
		20	5.10^{a} (±0.08)	4.24^{b} (±0.20)	4.10^{b} (±0.08)	-0.25*	14.4
		30	n/d	n/d	n/d	n/a	
		40	n/d	n/d	n/d	n/a	
Salmonella serovar Typhimu-rium	Manure spread on surface	10	6.30^{a} (±0.21)	5.91^{a} (±0.25)	6.00^{a} (±0.12)	-0.08*	60.0
		20	5.02^{a} (±0.40)	4.90^{a} (±0.36)	4.54^{a} (±0.31)	-0.12*	29.3
		30	n/d	n/d	n/d	n/a	
		40	n/d	n/d	n/d	n/a	
	Slurry injected into 10cm	10	5.51^{a} (±0.28)	5.27^{a} (±0.32)	4.93^{a} (±0.33)	-0.14*	28.6
		20	4.56^{a} (±0.17)	4.21^{a} (±0.26)	3.84^{a} (±0.22)	-0.18*	17.5
		30	n/d	n/d	n/d	n/a	
		40	n/d	n/d	n/d	n/a	

[a, b] significant ($P < 0.05$) differences among sampling time within each variable

* significant ($P < 0.05$) fit to a linear model

n/d below detection level (100 cfu gdw^{-1})

n/a not applicable

longer survival of the pathogens at the bottom of an unplanted soil profile compared to application of manure. However, in soil with lettuce plants, the pathogens did not move beyond the rooting depth after one simulated rainfall event. Moreover, the density of *E.*

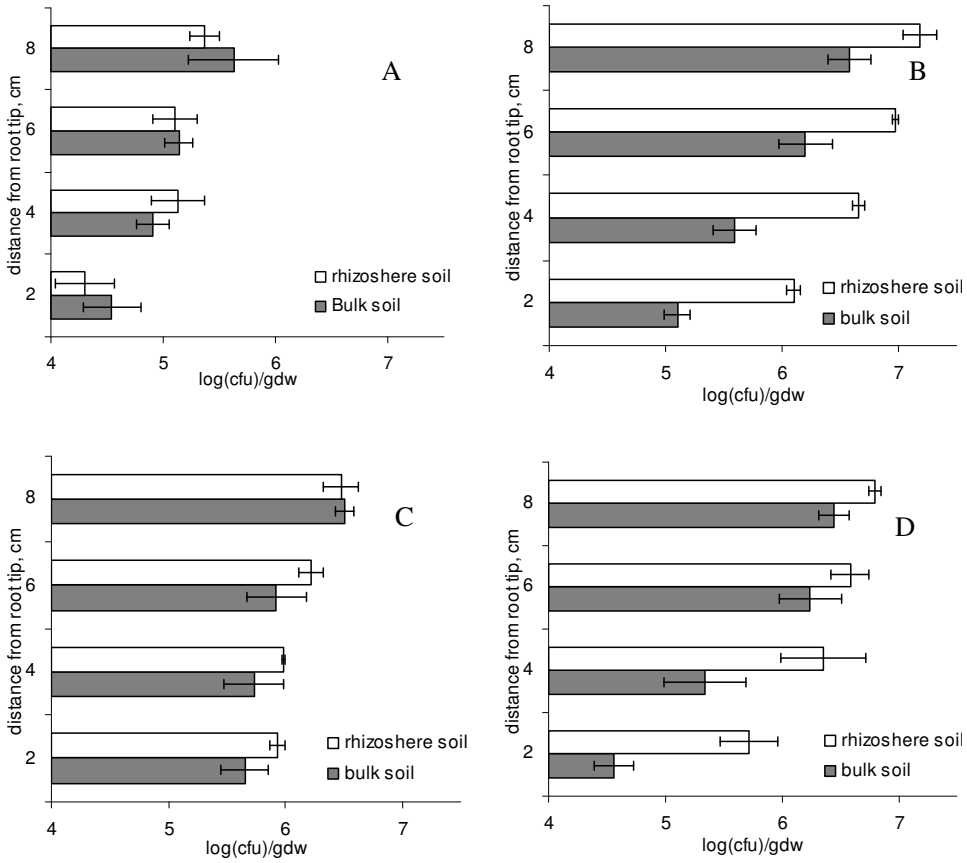

Fig. 7. Average density of *E. coli* O157:H7 (A, B) and *Salmonella* serovar Typhimurium (C, D) in the rhizosphere and bulk soil at a distance of 2, 4, 6 and 8 cm from the root tip in soil inoculated with manure spread on the soil surface (A, C) and slurry injected into 10cm of soil (B, D). Horizontal bars represent standard errors.

coli O157:H7 and *Salmonella* serovar Typhimurium was significantly higher in the rhizosphere of lettuce plants than in bulk soil after application of slurry, while this was not the case when manure was added.

The possibility of human pathogens to reach the ground water under different soil conditions had already been studied during previous experiments (Gagliardi and Karns, 2000; Ogden et al., 2001; Vinten et al., 2002; Mankin et al., 2007). Such soil factors as porosity, surface area, bulk density and macropore structure play an important role in the leaching potential of the introduced bacteria by their influence on adsorption and gravitational movement with water (van Elsas et al., 1991; Artz et al.,

2005). However, for organic substrates such as manure and slurry, the adsorption and desorption behavior of bacteria is not only due to differences in physical characteristics of the substrate, but also due to biophysical properties of the organic matter (Mankin et al., 2007). Experiments on the percolation of *Pseudomonas fluorescens* also showed that application of an adsorption substrate (such as bentonite clay) with to soil, may increase survival of *Pseudomonas fluorescens* and reduce transport to deeper layers (Hekman et al., 1994). In our experiments, it is likely that some of the organic matter in the slurry moved down the soil profile, while manure particles moved less readily. This may explain the higher pathogen concentrations at the bottom of the soil columns after application of slurry in comparison with farm-yard manure. Attachment of the pathogens to manure particles in the upper soil layers probably led to reduced percolation to deeper soil layers. Movement of nutrients as well as movement of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium may have been more pronounced in case of slurry application, so that the pathogens could survive better in lower soil layers in the presence of easily available compounds from slurry.

It was already reported before that *E. coli* O157:H7 can survive longer in soil in the presence of rye and alfalfa roots (Gagliardi and Karns, 2002). While manure and slurry characteristics such as chemical composition and microbial community have an important influence on survival of the pathogens, effect of the rhizosphere can also significantly affect survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium. During our experiments, the rhizosphere effect after slurry application seemed to be more pronounced, since slurry initially had 2 times higher concentrations of dissolved organic nitrogen and carbon compared to manure. The nitrate concentrations in the upper 10 cm of soil were also higher after slurry than manure application. This difference may enhance secretion of root exudates which could increase survival of the pathogens. High densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in the rhizosphere may increase the risk of internal contamination of plant products (Klerks et al., 2007).

In previous percolation studies with *Pseudomonas fluorescens* bacterial transport was dependent on the intensity of water flow and the number of water applications (Trevors et al., 1990). A limitation of our experiments was that water was applied only once (equal amount of water for all treatments) at one flow rate only. However, we were more interested in determining the influence of manure or slurry application method on the distribution and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soil columns. Another limitation was that relatively small soil columns were used, which may not be representative of field conditions due to the influence of soil cracks and wall effects on water flow (Hekman et al., 1994). However, only in such controlled experiments is it possible to distinguish the exact influence of certain factors. Moreover, for the soil columns that were used in our experiments, NMR analysis showed a uniform distribution of water in both horizontal and vertical

dimensions with insignificant wall effects and soil cracks. Thus, we assumed that our results were relatively realistic.

The obtained results suggest that there is a risk of contamination of surface and ground water with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium if slurry or manure is applied close to surface water. According to EU requirements for drinking water, no detectable faecal coliforms should be found in 100 ml of a water sample. In case of *E. coli* O157:H7, safety rules should be even more strict due to its very low infective dose (Chart, 2000). The highest risk of pathogen movement through the soil profile is immediately after slurry application, since even a small amount of rainfall can lead to drainage of water carrying significant numbers of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium to the ground water. This risk is less with farm-yard manure than slurry, as the pathogens were not detected at 40 cm depth within a couple of days after application. Moreover, the average survival time of *E. coli* O157:H7 at all depths in the soil columns was 1.67 times higher for treatments with slurry than with manure. These findings argue for surface application of farm-yard manure (often used at organic farms) rather than injection of slurry. At conventional farms, slurry is more often used than solid manure. In the Netherlands, injection of slurry is mandatory to reduce ammonia emission (Reijs et al., 2004), and this is practiced both in grasslands and arable fields (including vegetable fields). Unfortunately, this practice is associated with relatively high risks of percolation and survival of enteric pathogens in soil.

The presented research is part of a large research program (Franz et al., 2008; Klerks et al., 2007; Semenov et al., 2007; Semenov et al., 2008a) to quantify the risks of spread of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium from manure via soil to lettuce plants. The results will contribute to the development of a simulation model for the spread and survival of these two pathogens in soil and in the rhizosphere of lettuce plants after application of contaminated manure or slurry.

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Chapter 7

COLIWAVE a simulation model for survival of *E. coli* O157:H7 in dairy manure and manure-amended soil

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Abstract

A simulation model was developed to investigate the relative effects of temperature, oxygen concentration and substrate content on the oscillatory behaviour and survival of *E. coli* O157:H7 in manure and manure amended soil. The overall decline in *E. coli* O157:H7 was primarily determined by competition with autochthonous copiotrophic bacteria simulated by an inter-specific competition term according to Lotka-Volterra. Oscillations of bacterial populations were attained by the relationships between relative growth and death rates with readily available substrate content. The model contains a logistic and exponential relation of relative growth and death rates, respectively, of *E. coli* O157:H7 and copiotrophic bacteria with temperature, resulting in optimum curves for net growth rates similar to the curves reported in the literature. The model was used to perform sensitivity analysis and to evaluate different manure and soil management scenarios in terms of survival of *E. coli* O157:H7. The relative effects of changes in temperature on simulated survival time of *E. coli* O157:H7 were more pronounced than changes in oxygen condition. Testing manure storage scenarios with realistic data revealed that manure stored in a heap that was turned every week resulted in almost 70% reduction of *E. coli* O157:H7 survival compared to unturned manure. At the surface of a heap with unturned manure, simulated survival time was the longest (2.4 times longer than inside the same heap). The simulation model provides a new approach to investigating dynamic changes of invasive microorganisms in natural substrates such as manure or manure-amended soil.

Introduction

Modelling of microbial populations in artificial or natural substrates has become an important topic in ecology because it allows the estimation and prediction of microbial dynamics without performing long-term and often expensive experiments. One approach to model microbial populations is to use empirical models with the main aim to describe the observed data in the best possible way, using mathematical equations. Empirical models can be divided into primary and secondary models. Primary models predict microbial populations as a function of time under particular physiological and environmental conditions. The first primary model describing microbial growth was the exponential model (Monod, 1949). Although cells cannot multiply indefinitely, the exponential model with one parameter does not take a maximum cell density into account. Later, the 2-parameter logistic and 3-parameter Gompertz models were introduced into predictive microbiology to describe sigmoidal growth (Gibson et al., 1987). The modified Gompertz model (Zwietering et al., 1990), which includes the lag time, specific growth rate and maximum bacterial population, became the most widely used sigmoid function due to its flexibility in various conditions (Giannuzzi et al., 1998; McDonald and Sun, 1999). This Gompertz model was adjusted to become a more mechanistic growth model (Zwietering et al., 1994). Finally, various 4-parameter primary models were developed to describe microbial growth (Whiting and Cygnarowicz-Provost, 1992; Whiting and Bagi, 2002).

Secondary models describe the effect of environmental factors such as temperature, pH, water potential and substrate concentration on the growth of microorganisms. Monod was the first to model microbial growth in relation to environmental conditions, namely substrate concentration. The Monod model is a 2-parameter saturation curve taking substrate saturation and affinity into account. To model microbial growth in relation to various external variables, polynomial or response-surface models have often been used relating these variables to the parameters of the growth models mentioned above (Baranyi and Roberts, 1994; Bovill et al., 2000; Corradini and Peleg, 2006). After parameterisation, these models could empirically describe the effects of intrinsic factors of the growth medium (for example pH or water potential) and extrinsic factors imposed on the system (such as temperature or oxygen concentration) on the growth of an individual species in a specific substrate. The same mathematical approach to model development has also been adapted to model inactivation processes of bacteria (McDonald and Sun, 1999). However, these parameterized models are frequently not flexible enough to be used in other conditions. Moreover, in most empirical models competition between microorganisms and other microbial interactions are usually not considered (Oscar, 2007). Finally, empirical models are generally not suitable for describing the population dynamics under varying conditions.

The development of (semi-)mechanistic simulation models is a more complex approach, since these models attempt to include the relations between system components and the underlying biological and chemical processes that are responsible for the behaviour and dynamics of populations. Most of the existing models for microbial growth and survival were developed for sterile conditions (Blackburn et al., 1997; Duffy et al., 2000). Relatively few simulation models were developed to mimic the dynamics of various groups of microorganism in natural substrates such as soil or rhizosphere (Scott et al., 1995; van der Hoeven et al., 1996; Zelenev et al., 2000, 2005) or in different food commodities (Oscar, 2008).

In comparison with other simulation models, the BACWAVE model (Zelenev et al., 2000) is more realistic in that it mimics the oscillating dynamics of microbial populations. This feature is an important advantage since oscillations in microbial populations are a common phenomenon for both complex populations (Zelenev et al., 2005) as well as individual species (van Bruggen et al., 2008). Experiments in pure culture showed that oscillations were the result of intrinsic population dynamics (Vadasz et al., 2001) and were not induced by external factors, such as temperature changes. To detect the oscillations, frequent sampling over time is needed. In that case, fluctuations in populations can even be observed along sigmoidal decline curves (Messiha et al., 2007; Vidovic et al., 2007; Semenov et al., 2008a).

To our knowledge, the oscillating behaviour of declining microbial populations has not been modelled. Moreover, it is still unclear to what extent environmental variables may influence the oscillating dynamics of introduced bacteria in natural substrates. Combining the effects of different environmental factors and the innate microbial community on survival and oscillating dynamics of an introduced bacterial species in one simulation model may result in a better understanding of the observed survival patterns. Using a human pathogen as the object of simulation can contribute to our ability to predict the spread and survival of such a pathogen in the environment.

Escherichia coli O157:H7 was selected as a model species, since this pathogen has been responsible for a rising number of outbreaks and severe illnesses associated with the consumption of fresh produce during the last decade (Sivapalasingam et al., 2004). Cattle constitute the main reservoir of this pathogen, which can enter the food chain via contaminated manure and water (Franz et al., 2007a). The risk of produce contamination during the field stage is primarily determined by the survival capabilities of the pathogen in manure and manure-amended soil. Therefore, the influence of various biotic and abiotic variables on the survival of *E. coli* O157:H7 in manure and manure amended soil have been studied extensively (Kudva et al., 1998; Himathongkham et al., 1999; Nicholson et al., 2005; Semenov et al., 2007; Franz et al., 2008b; Semenov et al., 2008a), but not yet modelled. Thus, experimental data are available for parameterisation and validation of a simulation model. Temperature,

oxygen concentration, readily available substrate concentration and competition with autochthonous communities appear to be significant factors determining the persistence of *E. coli* O157:H7 in the farm environment (Franz et al., 2007a; Semenov et al., 2007; Franz et al., 2008b). Simulation of the survival of *E. coli* O157:H7 in manure and manure-amended soil under various environmental conditions could contribute to assessing the risk of vegetable contamination.

Consequently, the objectives of this study were 1) to develop a (semi-)mechanistic simulation model for oscillating dynamics of *E. coli* O157:H7 in competition with the autochthonous heterotrophic microbial communities in manure and manure-amended soil, 2) to investigate the relative effects of temperature, oxygen concentration and substrate content on the survival and oscillations of *E. coli* O157:H7 and 3) to evaluate different manure and soil management scenarios in terms of survival of *E. coli* O157:H7 in manure and manure-amended soil.

Materials and methods

Experimental data. For development of the model COLIWAVE the data on *E. coli* O157:H7 survival in manure (Semenov et al., 2007; Semenov et al., 2008a) and in manure-amended soil under different environmental conditions (Semenov et al., 2008b) were used. The model was validated with experimental data from parallel experiments (Franz et al., 2005; Franz et al., 2007a; Franz et al., 2008b). General characteristics for total copiotrophic bacteria and their dynamics were adapted from the model BACWAVE, which was constructed with experimental data on wave-like oscillations of copiotrophic bacteria in time and space (Zelenev et al., 2000).

Populations of *E. coli* O157:H7 and total copiotrophic bacteria (CFU/gdw) were expressed as µg of biomass carbon per cm^3 (µg C/cm^3) by using a transformation factor. This factor was 2.95×10^{-7} µg C gdw/CFU cm^3 for *E. coli* O157:H7, based on the size of one cell (2.4×10^{-12} cm^3/CFU) × the density of one cell (1.052 g/cm^3) × substrate density (1.3 gdw/cm^3) × dry weight of a cell (0.2 gdw/g) × carbon content of dry bacterial biomass (0.45 µg C/gdw). For total copiotrophic bacteria, the transformation factor was 6.15×10^{-8} µg C gdw/CFU cm^3, calculated from the size of one cell (1×10^{-12} cm^3/CFU) × density of one cell (1.052 g/cm^3) × substrate density (1.3 gdw/cm^3) × dry weight of cell (0.15 gdw/g) × carbon content of dry bacterial biomass (0.30 µg C/gdw) (Zelenev et al., 2000). Density of manure and soil amended with manure was assumed to be the same (1.3 gdw/cm^3) as well as carbon content of dry bacterial biomass (0.45 g C/gdw) in those substrates (Orskov, 1984; Uribelarrea et al., 1985).

Assumptions. It was assumed that *E. coli* O157:H7 populations would have the same physiological characteristics in sterilised and in fresh manure. Thus, the basic relative growth and death rates would be the same under these different conditions. The

population of E. coli O157:H7 would, however, decline faster in fresh manure due to competition with the autochthonous microbial community, assumed to be copiotrophic bacteria. Similar to the BACWAVE model (Zelenev et al., 2000), relations between relative growth and death rates are both dependent on substrate concentration according to a Monod and an inverse Monod function, respectively. These functions cross over at realistic substrate levels, so that oscillations in live bacteria and substrate appear over time.

We assumed two sources of substrate to be available for maintenance and growth by *E. coli* O157:H7 and copiotrophic bacteria in the model: dead cells which release carbon compounds by autolysis, and a constant background flux of substrate resulting from organic matter decomposition (Zelenev et al., 2000). Substrate from both sources was assumed to have the same composition and likelihood to be consumed by *E. coli* O157:H7 or copiotrophic bacteria with a certain yield coefficient. Only copiotrophic bacteria were considered as significant competitors of *E. coli* O157:H7, since they represent the largest part of the biomass in manure or manure-amended soil and have a similar ecological niche as *E. coli* O157:H7 in terms of carbon consumption. The level of readily available substrate (S) in the model was assumed to be equal to 1 – 2% of measured dissolved organic carbon (DOC) in manure or manure-amended soil (Zelenev et al., 2000). Although fresh manure and soil amended with manure are two different substrates in terms of autochthonous microbial community and levels of easily available substrate, we assumed that manure and soil would differ only in quantities of these variables.

The presence of oxygen was modelled at three possible levels: strictly anaerobic (0% of oxygen), semi-aerobic (1-3%) and aerobic (>15%). In contrast, temperature and substrate concentrations were included as continuous variables. Substrate concentrations are dynamic as a result of growth and death of *E. coli* O157:H7 and the microbial community, while temperature and oxygen concentration are external variables. It is assumed that the bacterial populations respond instantaneously to changes in environmental variables.

The influence of internal and external variables (substrate content and temperature or oxygen concentration) on the dynamics of the autochthonous microbial community is less pronounced than that of the pathogen, because the former consists of many species reacting differently to environmental conditions, resulting in shifts in the microbial composition (Semenov et al., 2007; Semenov et al., 2008a).

Model description. The model BACWAVE (Zelenev et al., 2000) was used as starting point, because fluctuations in populations of *E. coli* O157:H7 had been observed (Vidovic et al., 2007; Semenov et al., 2008b). The BACWAVE model was modified and extended to simulate the dynamics of introduced *E. coli* O157:H7 and of the autochthonous copiotrophic bacteria in manure and manure-amended soil. Lotka-

Volterra interaction terms for inter- and intraspecific competition between *E. coli* O157:H7 and the native copiotrophic bacteria were included (Hofbauer and Sigmund, 1988). The effects of temperature and availability of oxygen on the population dynamics were also added. A diagram of the model is presented in Fig. 1. Three ordinary differential equations were included in the model:

$$dX/dt = (\mu X(S) - DX(S)) \times X - B \times X^2 - C \times X \times Xtb; \quad (1)$$

$$dXtb/dt = (\mu Xtb(S) - DXtb(S)) \times Xtb - F \times Xtb^2 - G \times Xtb \times X; \quad (2)$$

$$dS/dt = -X \times \mu X(S)/YX + KXr \times X \times DX(S) - Xtb \times \mu Xtb(S)/YXtb + KXtb\_r \times Xtb \times DXtb(S) + BGF; \quad (3)$$

where

$$\mu X(S) = \mu Xmax \times S/(KXs + S), \quad (4)$$

$$DX(S) = DXmax \times KXd/(KXd + S), \quad (5)$$

$$\mu Xtb(S) = \mu Xtb\_max \times S/(KXtb\_s + S), \quad (6)$$

$$DXtb(S) = DXtb\_max \times KXtb\_d/(KXtb\_d + S), \quad (7)$$

t = time (h);
X = biomass of *E. coli* O157:H7, (µg C/cm^3);
µX(S) = relative growth rate of *E. coli* O157:H7, (h^{-1});
DX(S) = relative death rate of *E. coli* O157:H7, (h^{-1});
B = intra-specific competition for *E. coli* O157:H7, (cm^3/ µg C h^{-1});
C = inter-specific competition for *E. coli* O157:H7, (cm^3/ µg C h^{-1});
Xtb = biomass of total copiotrophic bacteria (µg C/cm^3);
µXtb(S) = relative growth rate of total copiotrophic bacteria, (h^{-1});
DXtb(S) = relative death rate of total copiotrophic bacteria, (h^{-1});
F = intra-specific competition for total copiotrophic bacteria, (cm^3/ µg C h^{-1});
G = inter-specific competition for total copiotrophic bacteria, (cm^3/ µg C h^{-1});
YX = yield coefficient for *E. coli* O157:H7, (µg C/ µg C);
KXr = recyclable fraction for *E. coli* O157:H7, (-);
YXtb = yield coefficient for total copiotrophic bacteria, (µg C/ µg C);
KXtb_r = recyclable fraction for total copiotrophic bacteria, (-);
BGF = constant background flux of substrate, (µg C/cm^3 h);
µXmax = Maximum relative growth rate for *E. coli* O157:H7, (h^{-1});
KXs = Substrate growth constant for *E. coli* O157:H7, (µg C/cm^3);
DXmax = Maximum relative death rate for *E. coli* O157:H7, (h^{-1});
KXd = Substrate death constant for *E. coli* O157:H7, (µg C/cm^3);
µXtb_max = Maximum relative growth rate for total copiotrophic bacteria, (h^{-1});
KXtb_s = Substrate growth constant for total copiotrophic bacteria, (µg C/cm^3);
DXtb_max = Maximum relative death rate for total copiotrophic bacteria, (h^{-1});
KXtb_d = Substrate death constant for total copiotrophic bacteria, (µg C/cm^3).

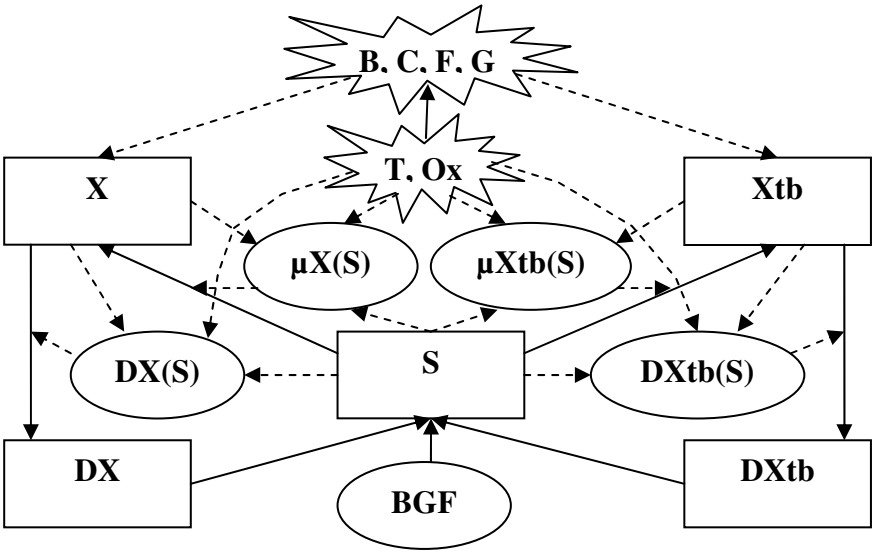

Fig. 1. Diagram of the simulation model with the state variables: X (biomass of *E. coli* O157:H7, μg C/cm^3), Xtb (biomass of total copiotrophic bacteria, μg C/cm^3), S (substrate, μg C/cm^3), DX (dead biomass of *E. coli* O157:H7, μg C/cm^3), DXtb (dead biomass of total copiotrophic bacteria, μg C/cm^3); rate variables: BGF (background flux, μg C/cm^3 h), DX(S) (relative death rate of *E. coli* O157:H7, h^{-1}), DXtb(S) (relative death rate of total copiotrophic bacteria, h^{-1}), μX(S) (relative growth rate of *E. coli* O157:H7, h^{-1}), μXtb(S) (relative growth rate of total copiotrophic bacteria, h^{-1}); parameters: T (temperature, Cº), Ox (availability of oxygen, binomial, unitless), B, C, F, G (competition parameters, unitless). Solid and dashed lines represent flows of matter and information, respectively.

Although the Lotka-Volterra interaction terms were subtracted from the net growth rates of *E. coli* O157:H7 and total copiotrophic bacteria (equations 1 and 2), these terms were not entered into the equation for change in substrate concentration (equation 3). The reason for this choice was the minimal impact of this interaction term on substrate content.

The COLIWAVE model was developed, optimised and validated in MATLAB (Version 7.0, The MathWorks, Inc.). The Runge-Kutta integration method was used with a flexible time step, which depends on intensity of changes in the model. The simulation model is available upon request.

Optimisation of parameters. The first step in parameter optimisation for simulation of *E. coli* O157:H7 dynamics in manure was the selection of a combination of values leading to the lowest sum of square errors (SSE) (with the highest regression coefficient R^2) between modelled and observed biomass of *E. coli* O157:H7 in sterilised manure (without inter-specific competition) over time (Semenov et al., 2007). The optimisation procedure resulted in parameterized empirical relations between temperature and characteristics of *E. coli* O157:H7 (yield coefficient (YX), intra-specific competition (B) and maximum relative growth (μXmax) and death rates (DXmax)), which were subsequently introduced into the model. A list with input parameters is presented in Table 1. Estimated substrate growth and death constants (KXs and KXd for *E. coli* O157:H7) were maintained as constants throughout the simulations.

When an acceptable fit ($R^2 > 0.9$) and significance level ($p<0.05$) of the regression of simulated *E. coli* O157:H7 biomass on observed data in sterilised manure was reached at all temperature levels, parameters responsible for total copiotrophic bacteria were optimised (Table 1). At this stage of optimisation, the effects of temperature and presence of oxygen on the inter-specific competition of the *E. coli* O157:H7 population with copiotrophic bacteria (C) , on the maximum relative growth and death rates (μ_Xtb_max and DXtb_max) and on the intra- and inter-specific competition of total copiotrophic bacteria (F and G) were calculated based on experimental data (Semenov et al., 2007; Semenov et al., 2008a) and introduced into the model.

Since a soil-manure mixture has a different autochthonous microbial community and average level of easily available substrate in comparison with fresh manure (Franz et al., 2008b; Semenov et al., 2008b), competition parameters both for *E. coli* O157:H7 and total copiotrophic bacteria (B, C, F and G) and background flux of substrate (BGF) were related to manure application rate (manure to soil ratio), based on experimental data with three manure to soil ratios (5%, 10% and 20%) (Semenov et al., 2008b).

Model validation. The simulation model of *E. coli* O157:H7 dynamics in manure was validated with the data from separate independent experiments on pathogen survival in manure (Franz et al., 2005; Franz et al., 2007b). Similarly, the model simulating survival of *E. coli* O157:H7 in soil amended with manure was validated with separate independent datasets (Franz et al., 2008b; Semenov et al., 2008b). Model performance was evaluated by regressing the observed versus predicted values and comparing the slope and intercept parameters against the 1:1 line (Pineiro et al., 2008).

Simulation experiments. The relative effect of temperature and oxygen content on the simulated survival time of *E. coli* O157:H7 in the model was assessed by varying these

Table 1. List and description of input parameters used in the model to simulate biomass of *E. coli* O157:H7 and copiotrophic bacteria in manure and soil amended with manure

Parameter	Variable	Value	Source
***E. coli* O157:H7**			
Maximum relative growth rate (h^{-1})	µXmax	0.01 – 0.20	(Semenov et al., 2007)[1]
Maximum relative death rate (h^{-1})	DXmax	0.04 – 0.60	(Semenov et al., 2007)[1]
Substrate growth constant (µg C / cm^3)	KXs	6.4	(Zelenev et al., 2000)
Substrate death constant (µg C / cm^3)	KXd	16	(Zelenev et al., 2000)
Yield coefficient (µg C / µg C)	YX	0.08 - 0.44	(Zelenev et al., 2000)
Recyclable fraction (unitless)	KXr	0.4	(Zelenev et al., 2000)
Intra-specific competition (cm^3 / µg C h^{-1})	B	1×10^{-6} - 1×10^{-3}	(Semenov et al., 2007)[1]
Inter-specific competition (cm^3 / µg C h^{-1})	C	2×10^{-5} - 1×10^{-2}	(Semenov et al., 2007)[1]
Biomass (µg C / cm^3)	X	2.5×10^{-4} - 2.5	(Semenov et al., 2007; Semenov et al., 2008a)[1]
Growth rate modifier (h^{-1})	µ_OxRate	0.7 – 1.1	(Semenov et al., 2008a)[1]
Death rate modifier (h^{-1})	D_OxRate	0.7 – 1.1	(Semenov et al., 2008a)[1]
Yield modifier (unitless)	Y_Ox	0.2 – 1.1	(Semenov et al., 2008a)[1]
Total copiotrophic bacteria			
Maximum relative growth rate (h^{-1})	µXtb_max	0.03 – 0.09	(Zelenev et al., 2000)
Maximum relative death rate (h^{-1})	DXtb_max	0.06 – 0.17	(Zelenev et al., 2000)
Substrate growth constant (µg C / cm^3)	KXtb_s	3	(Zelenev et al., 2000)
Substrate death constant (µg C / cm^3)	KXtb_d	14.5	(Zelenev et al., 2000)

Table 1 (continued)

Parameter	Variable	Value	Source
Yield coefficient (µg C / µg C)	YXtb	0.08 – 0.44	(Zelenev et al., 2000)
Recyclable fraction (unitless)	KXtb_r	0.4	(Zelenev et al., 2000)
Intra-specific competition (cm^3 / µg C h^{-1})	F	1×10^{-6} - 1×10^{-3}	(Semenov et al., 2007)[1]
Inter-specific competition (cm^3 / µg C h^{-1})	G	1×10^{-6} - 1×10^{-3}	(Semenov et al., 2007)[1]
Biomass (µg C / cm^3)	Xtb	5-100	(Semenov et al., 2007)[1]
Growth rate coefficient (h^{-1} T^{-1})	µ_OxRate_tb	0.7 – 1.1	(Semenov et al., 2008a)[1]
Death rate coefficient (h^{-1} T^{-1})	D_OxRate_tb	0.7 – 1.1	(Semenov et al., 2008a)[1]
Yield modifier (unitless)	Y_Ox_tb	0.2 – 1.1	(Semenov et al., 2008a)[1]
External values			
Temperature, (Cº)	T	5-60	(Semenov et al., 2007)
Oxygen (%)	Ox	0; 1.5; 18	(Semenov et al., 2008a)
Substrate (µg C/ cm^3)	S	2 - 150	(Zelenev et al., 2000; Semenov et al., 2007; Semenov et al., 2008a)
Background substrate flux (µg C/ cm^3 h)	BGF	0.2 - 5	(Zelenev et al., 2000)
Competition modifier (in relation to oxygen concentration) (cm^3 / µg C h^{-1})	Comp_Ox	0.9 – 13	(Semenov et al., 2008a)[1]
Competition modifier (for soil amended with manure) (unitless)	Comp_Soil	4 - 12	(Semenov et al., 2008b)[1]

[1] the source used for optimisation of this parameter

parameters around the mean. The influence of fluctuating vs. constant temperatures on survival and oscillations of *E. coli* O157:H7 was compared. In addition, different strategies of manure storage were evaluated by adapting the input parameters of the model. Survival times in turned and unturned manure heaps were simulated and compared to the baseline output by changing initial parameters of temperature and oxygen concentration.

Results

Population dynamics in sterile manure at different temperatures. The main biological parameters characterizing the *E. coli* O157:H7 population (μXmax, DXmax, KXs, KXd, YX, KXr and B) were first estimated (Table 1) by fitting the BACWAVE model to observed data of *E. coli* O157:H7 growth in sterilised manure at 16 ^{o}C (Semenov et al., 2007). The COLIWAVE model parameters for *E. coli* O157:H7 dynamics were then calibrated with similar data obtained at 7, 16, 23 and 33 ºC (Semenov et al., 2007) (Fig. 2A). When an acceptable goodness of fit was reached (average R^2 = 96%, $p<0.05$), the relations between temperature and maximum relative growth rate (μXmax), death rate (DXmax) and intra-specific competition (B) were determined. In order to get an optimum curve for the specific growth rate (actual growth rate – actual death rate) of *E. coli* (Heitzer et al., 1991), the maximum growth (μXmax) and death rate (DXmax) at different temperatures were fitted to a modified logistic and exponential equation, respectively, so that the observed specific growth rates fit the compound curve (Fig. 3). The optimization procedure resulted in the following empirical equations, which were subsequently included in the model:

$$\mu Xmax = 0.18/(1 + 1.05 \times \exp(-0.17 \times T + 3)) + 0.035, \quad (8)$$

$$DXmax = 0.09 \times \exp(0.05 \times T), \quad (9)$$

$$B = 10^{(0.07 \times T - 5.77)}. \quad (10)$$

At low substrate concentrations, the maximum relative growth and death rates of *E. coli* O157:H7 ranged from 0.01 to 0.12 h^{-1} and from 0.07 to 0.30 h^{-1}, respectively, depending on temperature (Table 1; Fig. 3 and 4), and resulted in net death. However, at relatively high substrate concentrations (in manure: S > 27 μg C/cm^3 and BGF > 2 μg C/cm^3 h^{-1}), the maximum growth and death rate varied from 0.04 to 0.2 h^{-1} and from 0.03 to 0.12 h^{-1} resulting in net growth of the *E. coli* O157:H7 population (Fig. 3 and 4).

Population dynamics in fresh manure at different temperatures. During the second step of model development, the population of total copiotrophic bacteria was introduced to mimic the influence of the autochthonous community on *E. coli* O157:H7 dynamics in manure or manure-amended soil. Although ecological parameters for total

copiotrophic bacteria were determined previously (Zelenev et al., 2000), they were optimized for our needs. Observed data in fresh manure (Semenov et al., 2007) was used to optimize the basic parameters as well as those for inter-specific competition between the autochthonous community and *E. coli* O157:H7 at different temperatures (7, 16, 23 and 33 ºC) (Fig. 2B). Optimization of *E. coli* O157:H7 dynamics in fresh manure in the presence of bacterial competitors resulted in the following additional empirical regression equations:

$$\mu Xtb\_max = 1.2 \times 10^{-3} \times T + 0.04, \quad (11)$$

$$DXtb\_max = 1.5 \times 10^{-3} \times T + 0.11, \quad (12)$$

$$C = 10^{(1.04 \times \ln(T) - 6.69)}, \quad (13)$$

$$F = 10^{(0.05 \times T - 5.64)}, \quad (14)$$

$$G = 10^{(0.05 \times T - 5.64)}. \quad (15)$$

At this stage of the simulation of survival of *E. coli* O157:H7 in fresh manure, the model was validated with two experimental data sets from parallel experiments (Franz et al., 2005; Franz et al., 2007a) (Fig. 5) resulting in a significant ($p < 0.05$) correlation ($R^2 = 0.93$ and 0.87, respectively) between observed and modelled data, with no significant deviation from the 1:1 line.

Population dynamics in fresh manure at different oxygen levels. During optimisation of *E. coli* O157:H7 dynamics in manure, the oxygen level was assumed to be semi-anaerobic (around 1.5%) (Semenov et al., 2007; Semenov et al., 2008a). To introduce two other oxygen conditions (aerobic and anaerobic, with 15 – 18% and ~0% oxygen, respectively), an additional set of parameters was added. Parameterization of µ_OxRate, D_OxRate, Y_Ox and Comp_Ox (Table 1) was done by fitting the existing model to sets of data for *E. coli* O157:H7 survival in aerobically and anaerobically stored manure at 16 ºC (Semenov et al., 2008a).

Population dynamics in manure-amended soil. The model obtained thus far was also optimized to simulate survival of *E. coli* O157:H7 in soil amended with manure (Fig. 6A). Initial parameters of available substrate (S), background flux (BGF) and biomass of total copiotrophic bacteria (Xtb) were adjusted to observed values in soil-manure mixtures (S = 2 – 10 µg C/cm^3; BGF = 0.2 – 1 µg C/cm^3 h and Xtb = 5 – 25 µg C/cm^3) (Franz et al., 2008b; Semenov et al., 2008b). Moreover, a competition modifier (Comp_Soil) was introduced to adjust intensity of inter and intra-specific competition (Table 1 and 2). Validation of the model for manure -amended soil showed a significant correlation between observed and predicted values, with no significant deviation from the 1:1 line (Fig. 6B; Fig. 7).

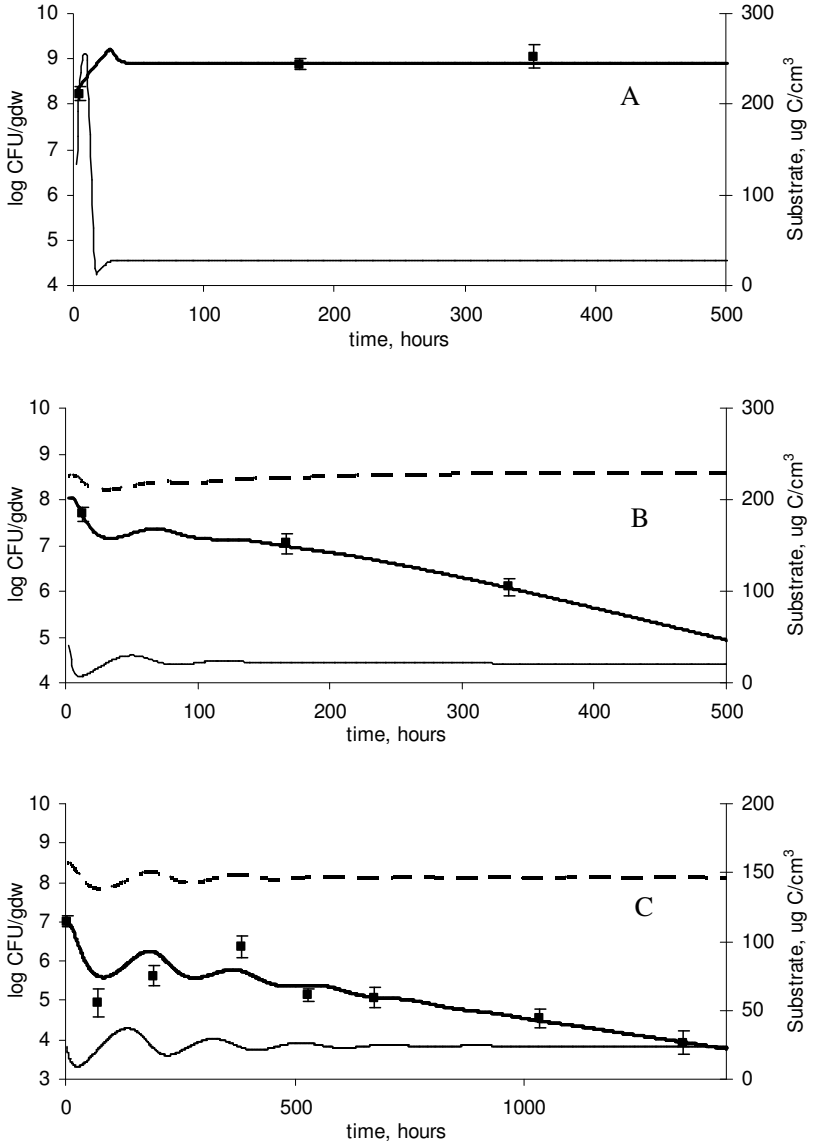

Fig. 2. Representative examples of simulated (solid bold line) and observed (closed squares) density of *E. coli* O157:H7, simulated total copiotrophic bacteria (dashed line) and available substrate (solid line) in sterilised manure at 23 °C (Semenov et al., 2007) (A); fresh manure at 23 °C (Semenov et al., 2007) (B) during calibration procedure and in fresh manure at 10 °C for a validation (Franz et al., 2005) (C).

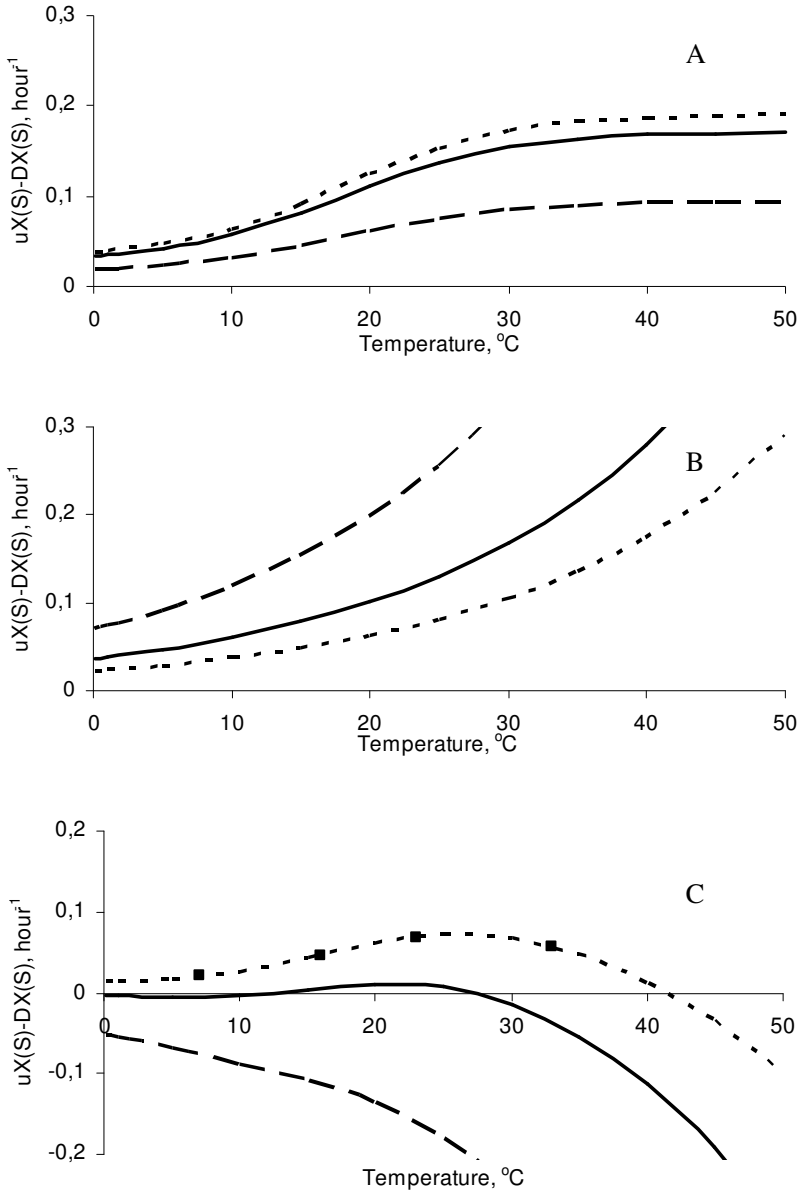

Fig. 3. Relation between temperature and relative growth rate (A); death rate (B); optimum curve (C) of *E. coli* O157:H7 population in manure at constant substrate content S = 25 µg C/cm^3 (striped line), S = 5 µg C/cm^3 (solid line) and at S = 50 µg C/cm^3 (dotted line). Closed squares represent observed data.

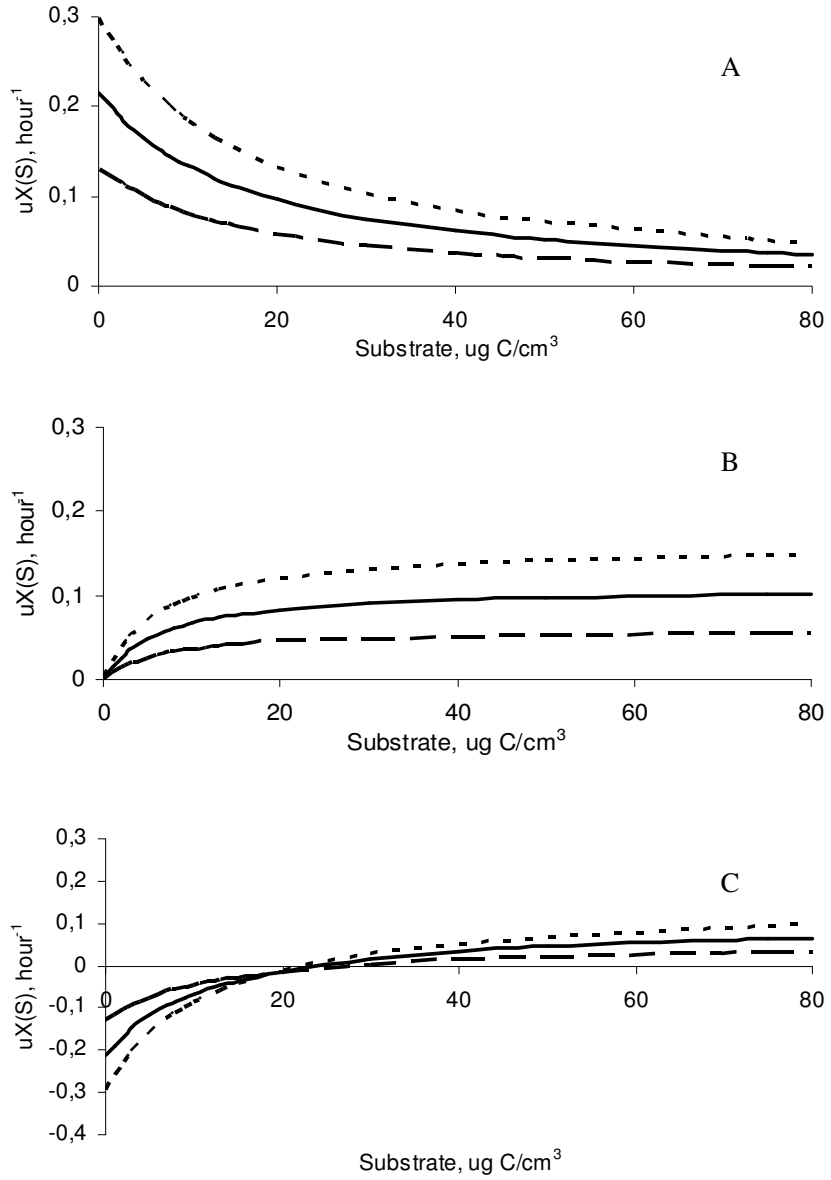

Fig. 4. Relation between substrate content and relative growth rate (A); death rate (B); optimum curve (C) of *E. coli* O157:H7 population in manure at constant temperatures T = 7 ºC (striped line), at T = 16 ºC (solid line) and at T = 23 ºC (dotted line).

Sensitivity analysis. Increasing the temperature by 5 ºC resulted in an average reduction of 20-25% of *E. coli* O157:H7 survival time for both manure and soil amended with manure (Table 3). Survival time of *E. coli* O157:H7 under fluctuating temperatures (± 7 ºC) was reduced by 15% in comparison with static temperatures with the same mean values (Table 4 and 5)(Fig. 8). The reduction in survival during aerobic storage was on average 83.8% of that under the baseline semi-aerobic conditions, while

Table 2. Best regression models for influence of temperature on characteristics of *E. coli* O157:H7 dynamics in manure and soil amended with manure.

Parameter	Equation
μXmax	$(0.18/(1 + 1.05 \times \exp(-0.17 \times T + 3)) + 0.035) \times \mu\_OxRate$
DXmax	$(0.09 \times \exp(0.05 \times T)) \times D\_OxRate$
μXtb_max	$(1.2 \times 10^{-3} \times T + 0.04) \times \mu\_OxRate\_tb$
DXtb_max	$(1.5 \times 10^{-3} \times T + 0.11) \times D\_OxRate\_tb$
YX	$0.4 \times Y\_Ox$
YXtb	$0.4 \times D\_Ox\_tb$
B	$10^{(0.07 \times T - 5.77)} \times Comp\_Ox \times Comp\_Soil$
C	$10^{(1.04 \times \ln(T) - 6.69)} \times Comp\_Ox \times Comp\_Soil$
F	$10^{(0.05 \times T - 5.64)} \times Comp\_Ox \times Comp\_Soil$
G	$10^{(0.05 \times T - 5.64)} \times Comp\_Ox \times Comp\_Soil$

under anaerobic conditions *E. coli* O157:H7 could survive 62 – 103% longer compared to the baseline (Table 3).

Comparison of different scenarios and manure storage methods. For the comparison of different scenarios for the simulation of *E. coli* O157:H7 survival in manure or soil amended with manure, initial parameters (T, Ox, S, BGF and Xtb) were adjusted to reflect realistic values of manure storage (Kudva et al., 1998; Nicholson et al., 2005; Semenov et al., 2008b). A reduction of the initial density of *E. coli* O157:H7 by 1 log CFU in manure and manure-amended soil resulted in an average 20-23% decrease in survival time (Table 4, 5). Simulation of *E. coli* O157:H7 survival with an initial density of 2-3 log CFU, which is the most probable and realistic density at a dairy farm in the Netherlands (Franz et al., 2008a) resulted in an average survival time of 12 – 30 days for manure and 4 – 20 days for soil amended with manure, respectively (Table 5). Survival of *E. coli* O157:H7 was longer when manure or soil-manure mixtures were more copiotrophic (relatively high BGF and substrate content + low density of total copiotrophic bacteria) compared to baseline. This situation is common at high-input conventional farms where slurry is used besides inorganic fertilizer (Reijs et al., 2004). On the other hand, oligotrophication of manure and soil amended with

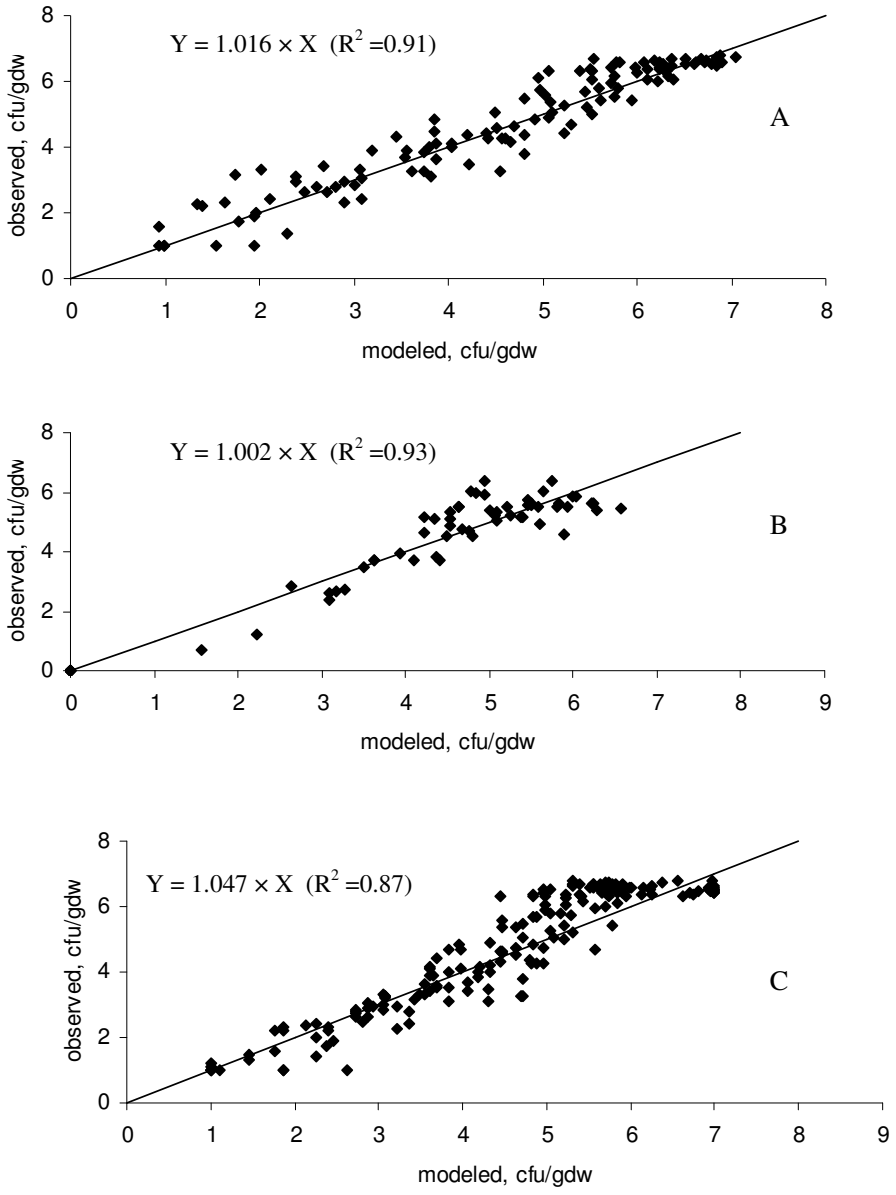

Fig. 5. Plot of the relation between observed and modelled density of *E. coli* O157:H7 in fresh manure (A) (Semenov et al., 2007) during calibration; in fresh manure (B) during validation (Franz et al., 2005) and in fresh manure (C) during validation (Franz et al., 2007a). Solid line represents the 1:1 line.

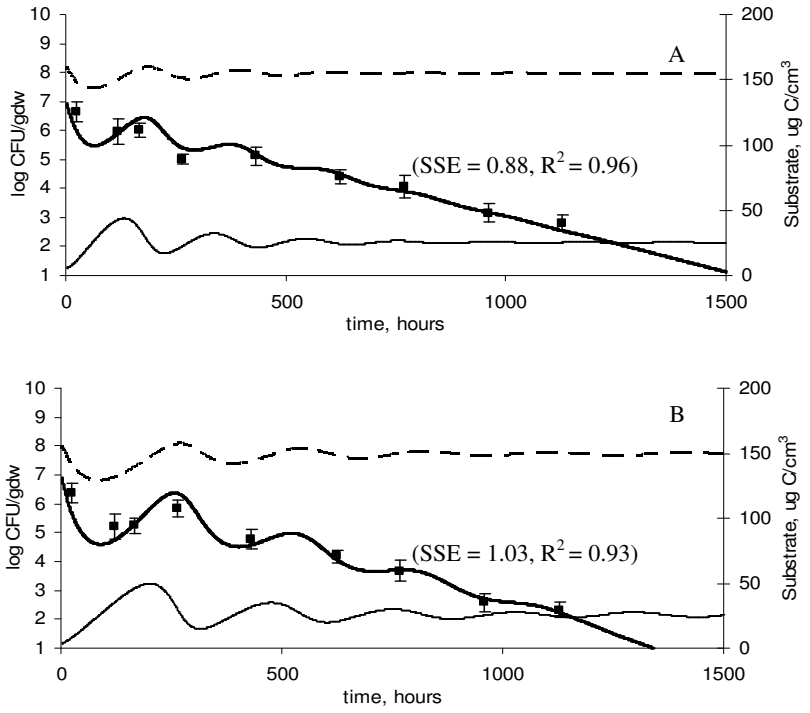

Fig. 6. Representative examples of simulated (solid bold line) and observed (closed squares) density of *E. coli* O157:H7, simulated total copiotrophic bacteria (dashed line) and available substrate (solid line) in soil amended with manure at 16 °C during calibration of parameters (Semenov et al., 2008b) (A) and in soil amended with manure at 16 °C during validation (Franz et al., 2008b) (B).

manure reduced the survival time of *E. coli* O157:H7 by 14% and 44%, respectively. Increasing the initial density of total copiotrophic bacteria by1 log CFU (without changes in S and BGF) resulted in 24 – 38% survival time reduction (as a result of higher competition).

Additionally, the simulation model was run for two manure storage strategies (Table 6). Manure stored in a heap which was turned every week (alternating oxic conditions with 15-18 % O_2 for 1 day at temperature (20 – 25 ºC) and anoxic conditions with 0 % O_2 for 6 days combined with a continuous rise in temperature to 40 ºC during one week) resulted in almost 70% reduction of *E. coli* O157:H7 survival (~ 8 days) compared to unturned manure (~ 26 days). At the surface of a heap with unturned manure (semi-aerobic conditions at 15 ± 10 °C), simulated survival time was 2.4 times longer (~63 days) than inside of the same heap (~26 days) and almost 8 times longer than in the heap with turned manure (~8 days).

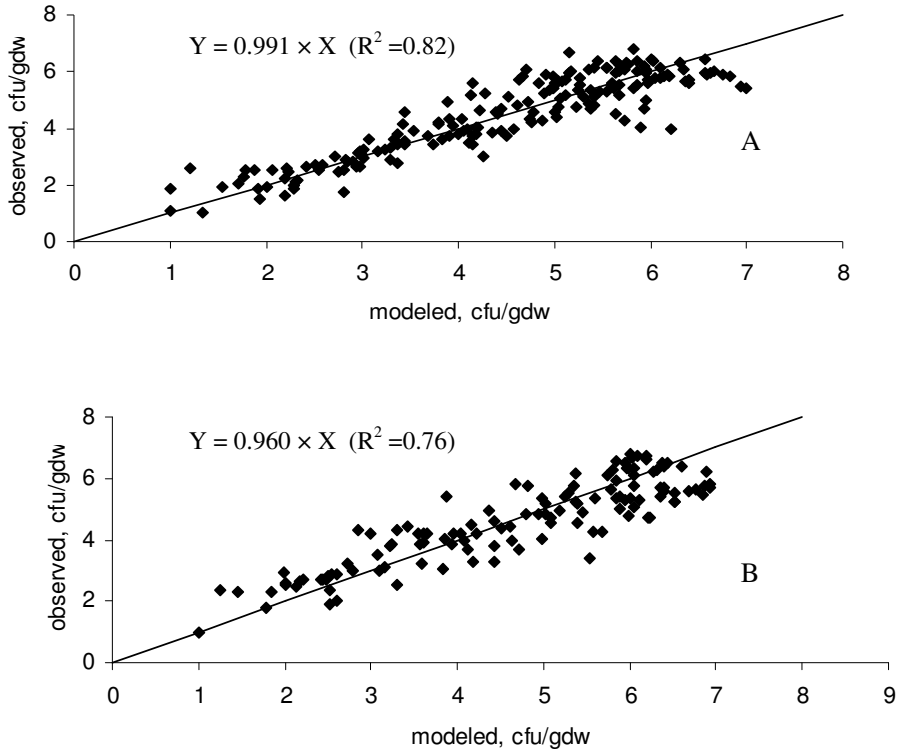

Fig. 7. Plot of the correlation between observed and modelled density of *E. coli* O157:H7 in soil amended with manure (Semenov et al., 2008b) (A) during calibration and in soil amended with manure (B) during validation (Franz et al., 2008b). Solid line represents the 1:1 line.

Discussion

Model performance and major outcomes. The main objective of this study was to develop a mechanistic simulation model for oscillating dynamics of *E. coli* O157:H7 in competition with the heterotrophic microbial communities in manure and manure-amended soil in response to different temperatures, oxygen concentrations and substrate contents. Indeed, the model COLIWAVE was able to mimic oscillations in populations of *E. coli* O157:H7 as observed in the beginning of typical survival curves (Vidovic et al., 2007; Semenov et al., 2008b). These oscillations were attained by the relationships between relative growth and death rates with readily available substrate content, similar to results obtained with the BACWAVE model (Zelenev et al., 2000). The ability of the model to simulate wave-like fluctuations resulted in a 10 to 15%

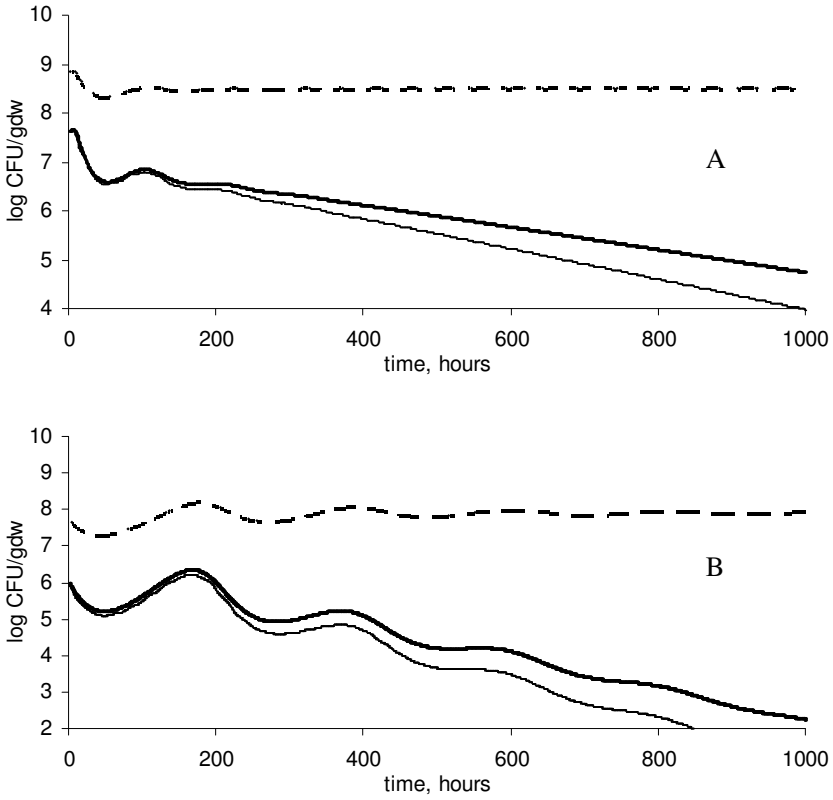

Fig. 8. Representative examples of simulated (solid lines) density of *E. coli* O157:H7 and simulated total copiotrophic bacteria (dashed line) in manure (A) and soil amended with manure (B) at 16 °C (solid bold line) and at 16 ± 7 °C (solid line).

better fit to observed data as determined by the sum of squares error compared to that of the Weibull model (Franz et al., 2008b). Therefore, we expect a better prediction of survival capacities of the enteropathogen by the COLIWAVE model.

The death rates used in COLIWAVE are, by themselves, not large enough to mimic the fast decline observed in nonsterile manure or soil, possibly because the stress encountered by the sudden change in substrate is not taken into account. Intra-specific competition and inter-specific competition with the heterotrophic autochthonous microbial community largely determined the overall decline in *E. coli* O157:H7. In sterile manure, the *E. coli* O157:H7 population initially increased and then declined only very slowly, both in the model and in reality (Semenov et al., 2007). Inter- and intra-specific competition were modelled according Lotka-Volterra interaction terms. Removal of biomass through competition by a reduction from the net

Table 3. Results of the sensitivity analysis on the simulated survival time of *E. coli* O157:H7 in manure and in soil amended with manure

	Parameter values[1]	Simulated survival time needed to reach detection limit[2], days	Survival time reduction, %
Manure			
Baseline values	X = 0.25 µg C/cm^3 (~ 6 log CFU/gdw); Xtb = 30 µg C/cm^3 (~ 8.7 log CFU/gdw); T = 16 °C; Ox =1.5%; S = 50 µg C/cm^3; BGF = 2 µg C/cm^3.	83.25	-
Aerobic conditions	Ox = 18%.	13.07	- 84.3
Anaerobic conditions	Ox = 0%.	135.39	+ 62.6
Low temperature	T = 10 °C	102.67	+ 23.3
Low temperature	T = 15 °C	85.17	+ 2.3
Medium temperature	T = 20 °C.	62.20	- 25.3
Medium temperature	T = 25 °C.	21.08	- 74.7
High temperature	T = 30 °C.	9.05	- 89.1
High temperature	T = 35 °C.	5.04	- 93.9
Soil amended with manure			
Baseline	X = 0.25 µg C/cm^3 (~ 6 log CFU/gdw); Xtb = 7 µg C/cm^3 (~ 8 log CFU/gdw); T = 16 °C; Ox =1.5%; S = 7 µg C/cm^3; BGF = 0.5 µg C/cm^3.	54.16	-
Aerobic conditions	Ox = 18%.	9.06	- 83.3
Anaerobic conditions	Ox = 0%.	110.42	+ 103.9
Low temperature	T = 10 °C	63.26	+ 16.8
Low temperature	T = 15 °C	56.19	+ 3.7
Medium temperature	T = 20 °C.	39.11	- 27.8
Medium temperature	T = 25 °C.	17.06	- 68.5
High temperature	T = 30 °C.	9.05	- 83.3
High temperature	T = 35 °C.	4.09	- 92.4

[1] abbreviations of the parameters are presented in Table 1.

[2] 1 log CFU/gdw

Table 4. Results of different scenarios on the simulated survival time of *E. coli* O157:H7 in manure

	Parameter values[1]	Simulated survival time needed to reach detection limit[2], days	Survival time reduction, %
Baseline values	X = 0.25 µg C/cm^3 (~ 6 log CFU/gdw); Xtb = 30 µg C/cm^3 (~ 8.7 log CFU/gdw); T = 16 °C; Ox = 1.5%; S = 50 µg C/cm^3; BGF = 2 µg C/cm^3.	83.25	-
Baseline density of *E. coli* O157:H7 -1 log CFU	X = 2.5 × 10^{-2} µg C/cm^3 (~ 5 log CFU/gdw).	65.20	- 21.7
Baseline density of *E. coli* O157:H7 -2 log CFU	X = 2.5 × 10^{-3} µg C/cm^3 (~ 4 log CFU/gdw).	48.15	- 42.2
Baseline density of *E. coli* O157:H7 -3 log CFU	X = 2.5 × 10^{-4} µg C/cm^3 (~ 3 log CFU/gdw).	30.11	- 63.8
Baseline density of *E. coli* O157:H7 -4 log CFU	X = 2.5 × 10^{-5} µg C/cm^3 (~ 2 log CFU/gdw).	12.06	- 85.5
Daily temperature fluctuation	T = 16 ± 7 °C	70.26	- 15.6
Oligotrophic conditions	S / Xtb = 0.2 (S = 20 µg C/cm^3; BGF = 1 µg C/cm^3; Xtb = 100 µg C/cm^3).	71.17	- 14.5
Copiotrophic conditions	S / Xtb = 10 (S = 100 µg C/cm^3; BGF = 3 µg C/cm^3; Xtb = 10 µg C/cm^3).	95.15	+ 14.3
High density of total copiotrophic bacteria +1 log CFU	Xtb = 300 µg C/cm^3 (~ 9.6 log CFU/gdw)	51.15	- 38.6
Low density of total copiotrophic bacteria -1 log CFU	Xtb = 3 µg C/cm^3 (~ 7.7 log CFU/gdw)	118.34	+ 42.2

[1] abbreviations of the parameters are presented in Table 1.

[2] 1 log CFU/gdw

Table 5. Results of different scenarios on the simulated survival time of *E. coli* O157:H7 in soil amended with manure

	Parameter values[1]	Simulated survival time needed to reach detection limit[2], days	Survival time reduction, %
Baseline values	X = 0.25 µg C/cm^3 (~ 6 log CFU/gdw); Xtb = 7 µg C/cm^3 (~ 8 log CFU/gdw); T = 16 °C; Ox = 1.5%; S = 7 µg C/cm^3; BGF = 0.5 µg C/cm^3.	54.16	-
Baseline density of *E. coli* O157:H7 -1 log CFU	X = 2.5 × 10^{-2} µg C/cm^3 (~ 5 log CFU/gdw).	43.13	- 20.4
Baseline density of *E. coli* O157:H7 -2 log CFU	X = 2.5 × 10^{-3} µg C/cm^3 (~ 4 log CFU/gdw).	31.10	- 42.6
Baseline density of *E. coli* O157:H7 -3 log CFU	X = 2.5 × 10^{-4} µg C/cm^3 (~ 3 log CFU/gdw).	20.09	- 62.9
Baseline density of *E. coli* O157:H7 -4 log CFU	X = 2.5 × 10^{-5} µg C/cm^3 (~ 2 log CFU/gdw).	4.18	- 92.3
Daily temperature fluctuation	T = 16 ± 7 °C	46.20	- 14.7
Oligotrophic conditions	S / Xtb = 0.2 (S = 3 µg C/cm^3; BGF = 0.25 µg C/cm^3; Xtb = 15 µg C/cm^3).	30.11	- 44.4
Copiotrophic conditions	S / Xtb = 10 (S = 30 µg C/cm^3; BGF = 1 µg C/cm^3; Xtb = 3 µg C/cm^3).	79.28	+ 46.4
High density of total copiotrophic bacteria +1 log CFU	Xtb = 30 µg C/cm^3 (~ 9.6 log CFU/gdw)	41.13	-24.1
Low density of total copiotrophic bacteria -1 log CFU	Xtb = 3 µg C/cm^3 (~ 7.7 log CFU/gdw)	62.19	14.8

[1] abbreviations of the parameters are presented in Table 1.

[2] 1 log CFU/gdw

specific growth rates of *E. coli* O157:H7 and the microbial community (equations 1 and 2) is not accompanied by an increase in the substrate content in COLIWAVE. The reasons are that not all competing cells would die (but probably become dormant instead), and addition of the competing biomass to the substrate content in equation 3 did not change the overall behaviour of *E. coli* O157:H7 nor of the autochthonous microbial community (data not shown).

Nevertheless, significant contributions to our understanding of population dynamics are made with COLIWAVE. The most important is the development of a method to estimate relative growth and death rates of *E. coli* O157:H7 and the autochthonous microbial community in relation to substrate as well as to temperature. The net relative growth rates, as calculated from observed changes in population densities, are related to substrate and temperature in the form of a saturation curve (Fig. 4) and a typical optimum curve (Fig. 3), respectively. Relations of apparent relative growth rates to temperature have been described by various equations, such as the Arrhenius equation (Davey, 1989), square root (Ratkowsky et al., 1982), cardinal (Rosso et al., 1995) and polynomial models. However, it is likely that these equations reflect our inability to distinguish growth and death directly. In reality the physiological responses of bacterial cells resulting in growth or death are probably different in relation to temperature (and other environmental factors). COLIWAVE contains a logistic and exponential relation between temperature and the *E. coli* O157:H7 relative growth and death rate, respectively. After combining relative growth and death rates the resulting optimum curve was similar to the curves reported in the literature (Heitzer et al., 1991; Scott et al., 1995). In the presence of competition, however, the optimum shifts to much lower temperatures, due to increased sensitivity of *E. coli* O157:H7 to competition at higher temperatures (Semenov et al., 2007). Shifts in the composition of the autochthonous microbial community at changing temperatures could be responsible for the relative insensitivity of the whole community to competition at various temperatures.

In various simulated conditions, the maximum relative growth rate for *E. coli* O157:H7 varied from 0.01 to 0.2 h^{-1}, depended on temperature, oxygen concentration and easily available substrate. Experiments in sterile nutrient broth showed that the maximum relative growth rate could be more than 10 times higher (Heitzer et al., 1991). In natural substrates such as soil and manure, however, the actual relative growth rate can be much lower due to the complexity of carbon compounds with different decomposition rates and the heterogeneity of the distribution of nutrients resulting in a mix of high and low net growth rates (Semenov et al., 2007; Semenov et al., 2008a). In empirical models based on bacterial dynamics in broth, continuous death (besides growth) has usually been neglected, while in our model the actual relative death rate was higher than the growth rate and it was dependent on environmental

Table 6. Results of the effect of different storage strategies of manure on the simulated survival time of *E. coli* O157:H7

Description of storage strategies of manure	Characteristics of the storage[1]	Simulated survival time needed to reach detection limit[2], days	Survival time reduction, %
Baseline	X = 0.25 µg C/cm^3 (~ 6 log CFU/gdw); Xtb = 30 µg C/cm^3 (~ 8.7 log CFU/gdw); T = 16 °C; Ox = 1.5%; S = 50 µg C/cm^3; BGF = 2 µg C/cm^3.	83.25	-
Unturned manure stored in a heap (anaerobic condition, temperature increases from 10 °C to 40 °C during first week with following temperature fluctuations around 40 °C (±5 °C))	T = 10 - 45 °C; Ox =0%.	26.11	- 68.64
Turned manure stored in a heap (aerobic condition for one day every week, temperature increases from 10 °C to 40 °C during 6 days and decreasing (to 20 - 25 °C) during manure turning (1 day).	T = 10 - 45 °C; Ox = 0% (18% every 7 days).	8.13	- 90.23
Unturned manure stored in a heap (surface) (semi-aerobic condition, temperature fluctuations around 15 °C (±10 °C))	T = 5 - 25 °C; Ox = 1.5%.	63.54	- 23.68

[1] abbreviations of the parameters are presented in Table 1.

[2] 1 log CFU/gdw

conditions. Although no direct experimental data have been found to verify the range of death rates in COLIWAVE (0.04 to 0.60 h^{-1}), the same magnitudes have been used or death rates in several previous studies (Scott et al., 1995; Zelenev et al., 2000, 2005).

A second important objective was to investigate the relative effects of temperature, oxygen concentration and substrate content on the behavior of *E. coli* O157:H7 populations in manure and manure-amended soil. Separate relationships of the relative growth and death rates with temperature resulted in good fits of the simulated *E. coli* O157:H7 and copiotrophic bacterial populations to the observed data over time at various temperatures. The effects of diurnally oscillating compared to constant temperatures on simulated populations and estimated survival times were similar to those observed (Semenov et al., 2007), namely decreased survival times with increasing amplitudes of the temperature oscillations (Tables 4 and 5). No artificial delays in physiological response time were needed to attain these results, indicating that the nonlinearity of the relations between relative growth and death rates with temperature were sufficient to explain the reduction in survival time under oscillating temperatures (Scherm and van Bruggen, 1994). Oscillating temperatures hardly affected the oscillations in the pathogen populations, which come about by the interaction with substrate and are not determined by oscillations in external environmental conditions.

E. coli O157:H7 is a facultative anaerobic organism and although relative growth is less intensive in anaerobic conditions as compared to aerobic conditions, the pathogen can survive better in anaerobic conditions (Kudva et al., 1998; Semenov et al., 2008a). There is probably a complex influence of oxygen conditions on pathogen behaviour through a combination of interactions between oxygen availability and physiological processes, decomposition of organic matter (and the associated chemical changes in the substrate) and changes in the autochthonous microbial community. Therefore, an additional parameter was introduced into the model to adapt competition, consumption of nutrients and relative growth and death rates to discrete oxygen conditions.

The relative effects of changes in temperature on simulated survival time of *E. coli* O157:H7 were much greater (20 fold difference between 10 and 30^{o}C) than changes in oxygen condition (10 fold difference between aerobic and anaerobic conditions). Effects of substrate availability are difficult to evaluate as such, since this is always associated with differences in competing bacterial populations and in background flux as affected by decomposition of organic matter. These combined effects were evaluated in the scenario studies carried out with the model.

A third objective was to evaluate different manure and soil management scenarios in terms of survival of *E. coli* O157:H7 in manure and manure-amended soil. Effects of initial concentrations of *E. coli* O157:H7 in manure and soil were much greater than the effects of realistic changes in substrate availability (combined differences in the ratio of substrate concentration and populations of heterotrophic autochthonous community and background flux) on the ultimate survival time of the pathogen (Tables 4 and 5). Thus, it seems important to prevent high initial

concentrations of *E. coli* O157:H7 in manure, for example by feeding cows with a high fiber diet (Franz et al., 2005; Franz et al., 2007a). High initial populations of competing bacteria are more influential for pathogen survival in manure than in soil, due to the greater substrate availability in manure. Finally, turning a manure heap reduces the estimated survival time of *E. coli* O157:H7 more than leaving the heap unturned. Survival of the pathogen was longest at the surface of an unturned manure heap. Thus, the model allowed estimation of the consequences of different manure and soil management strategies and the effectiveness of potential control strategies.

Comparison with other models. To our knowledge, this is the first simulation model to describe the survival dynamics of a human enteric pathogen in natural substrates. COLIWAVE is a flexible model to study the survival of *E. coli* O157:H7 in different environmental conditions and can be applied to a wide range of management scenarios or even adapted for similar pathogens (*Listeria spp.*, *Salmonella spp.*, etc.). All previous attempts were mainly empirical models based on mathematical relations between biotic and abiotic factors and growth or survival of certain microorganisms (Gibson et al., 1987; Zwietering et al., 1990; Whiting and Cygnarowicz-Provost, 1992). In comparison with other simulation models that calculate dynamics and behaviour of heterotrophic microorganisms (De Ruiter, 1993; Grant et al., 1993; Katterer and Andren, 2001), our model separated the relative rate of change in relative growth and death rates for all populations included in the model. The BACWAVE model (Zelenev et al., 2000) which was used as a basis for the COLIWAVE model did not take into account the influence of changing extrinsic factors such as temperature and oxygen concentration.

Limitations of the model. Although the model can describe patterns of *E. coli* O157:H7 dynamics in various environmental conditions under the influence of the most important factors, it is still constrained by several assumptions and limitations. First of all, inter- and intraspecific competition is modeled by empirical Lotka-Volterra terms. These terms do not take the mechanisms of competition into account (Svirezhev, 2008), although competitive interactions are satisfactorily described (Svirezhev, 2008). However, the assumption of a homogeneous distribution of substrate and microorganisms is not valid at low substrate concentrations and low densities of bacteria, while the actual distribution of low numbers of cells is very important under natural heterogeneous conditions. In COLIWAVE the reduction in *E. coli* O157:H7 biomass due to competition with the relatively stable biomass of total copiotrophic bacteria has the same magnitude at low *E. coli* O157:H7 densities (1 – 2 log CFU) as at higher densities (2 – 8 log CFU), so that a lower asymptote is not modeled for the decline in *E. coli* O157:H7 populations. Simulated pathogen survival at low population densities (<1 – 2 log CFU) may therefore not be reliable. The model could be improved

by replacing the empirical competition terms (Lotka-Volterra relations) by more mechanistic components. Separation of each population into active and dormant cells might lead to a more realistic simulation of the competition for substrate. Moreover, division of the autochthonous microbial community over copiotrophic and oligotrophic bacteria would lead to a lower asymptote in the *E. coli* O157:H7 survival curve as the density of copiotrophic bacteria (main competitors of *E. coli* O157:H7 for substrate) would decrease and that of oligotrophs would increase over time (Zelenev et al., 2006). Moreover, the constant background flux of substrate (BGF) could be replaced by an organic matter decomposition model as in the model BACWAVE-WEB (Zelenev et al., 2006).

Influence of oxygen in the model was simulated with three levels: anaerobic (0% of oxygen), semi-aerobic (1-3%) and aerobic (>15%). All previous experiments studying the influence of oxygen on survival of *E. coli* O157:H7 in natural substrates (Kudva et al., 1998; Nicholson et al., 2005; Semenov et al., 2008a) had only two oxygen conditions: aerobic and anaerobic. Therefore, no experimental data was available to model influence of continuous oxygen changes on survival of the pathogen.

Finally, the behaviour of *E. coli* O157:H7 at negative temperatures in manure or manure-amended soil was not included in the model. Although, in some parts of the world the temperature could be below zero for significant periods of time, seasonal temperature variations in soil (Stoller and Wax, 1973) and manure (Nicholson *et al.*, 2005) are in most cases above zero. The model could be improved by adding additional environmental conditions such as continuous oxygen changes, negative temperatures, as well as the influence of other environmental characteristics (e.g. pH, water content, or a_w).

Conclusions. The simulation model described in this study provides a new approach to investigating dynamic changes of invasive microorganisms in natural substrates as affected by various environmental factors and interactions with the autochthonous microbial community. Separate calculation of growth and death rates combined with Lotka-Volterra competition terms resulted in a (semi-) mechanistic explanation of pathogen behaviour and decline in manure and manure-amended soil. The model is flexible enough to simulate complex scenarios of manure storage and its application to soil. Estimated survival times were similar to those observed in previous studies (Kudva et al., 1998; Himathongkham et al., 1999; Nicholson et al., 2005; Fremaux et al., 2007). This model will help to identify and evaluate potential strategies for control of enteric pathogen in the vegetable production chain without the cost and restrictions of experimental studies.

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Chapter 8

General Discussion

Introduction

The number of outbreaks and severe illnesses associated with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium has been increasing in recent years (Beuchat, 2002) (Sivapalasingam et al., 2004). Although cow manure and slurry are known as a natural reservoir of these enteropathogens, both types of animal feces are intensively used as a soil fertilizer during the primary production phase of vegetables. Enteric pathogens associated with manure or slurry can spread onto vegetable plants through splash dispersal by rain or irrigation water (Boyer, 2008). These pathogens can also be taken up by the roots and become internalized, (Franz et al., 2007; Klerks et al., 2007), so that they cannot be removed by various post-harvest decontamination procedures (Niemira, 2008). Moreover, some of these procedures may not be allowed in particular agricultural production chains (e.g. usage of chemicals on organic produce). Therefore, it becomes evident that prevention of pre-harvest contamination by identifying the risk factors responsible for spread and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium as well as by prediction of pathogen behavior in different agricultural habitats is of high importance.

Most experiments on survival of enteropathogens in manure, slurry, soil and on (in) plants had focused on the enteropathogens *per se* without paying much attention to possible relations between their survival and different external environmental characteristics or microbial communities (Guan and Holley, 2003). Since both spread and survival of enteropathogens likely depend on interactions between various environmental factors, the overall set of abiotic and biotic environmental characteristics should be taken into account, which could best be done by simulation modelling. Accordingly, the focus of this thesis was to identify factors that can minimize pathogen spread, multiplication and survival in primary plant production chains where manure is used to fertilize the soil, as well as to develop a simulation model for prediction of enteropathogen survival in manure and soil amended with manure under different management scenarios.

In this chapter major contributions and limitations of the performed research are presented. Moreover, recommendations for future research as well as potential implications of the results are discussed at the end.

Major contributions

Behavior of pathogen populations in relation to substrate. The influence of environmental factors on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in natural substrates can be correctly interpreted only when their population behavior is understood. Thus, knowledge about short-term population changes in relation to easily degradable substrate in manure or soil is needed to interpret effects of temperature and farming system on the risk of survival of these pathogens in these systems. In this thesis *E. coli* O157:H7 introduced into soil-manure

mixtures declined with irregular fluctuations (Chapter 5), contrary to the generally accepted monotonous decline. Similar fluctuations in introduced populations of plant and human pathogens along sigmoidal decline curves could be gleaned from graphs presented previously (Messiha, 2006; Vidovic et al., 2007). The fluctuations in *E. coli* O157:H7 populations were strongly affected by the predominant trophic group of bacteria (copiotrophic versus oligotrophic) in a particular habitat (Chapter 5), indicating the importance of easily degradable substrate concentrations. The observed fluctuations around the survival curve were simulated by the interactions between growth and death rates with easily degradable substrate in manure and soil (Chapter 7). The instability of the introduced *E. coli* O157:H7 populations around the survival curves, as measured by approximate entropy (Chapter 5), proved to be smaller in organically than in conventionally managed soils and could form an indication of the instability of the soils themselves.

The relative density of copiotrophic and oligotrophic bacteria was the most important factor responsible for the intensity of population fluctuations over time. Oligotrophs are better able to live in nutrient-poor environments, are characterized by relatively slow growth rates and less extreme temporal changes in populations in comparison to copiotrophs (Semenov, 1991; Zelenev et al., 2006). One of the main ecological functions of oligotrophic bacteria was thought to be a dampening effect on fluctuations in copiotrophic populations (Zelenev et al., 2006). This assumption is corroborated by the less irregular (more stable) population decline of *E. coli* O157:H7 at higher ratios of oligotrophic to copiotrophic bacteria. Thus, oligotrophication of substrates carrying enteropathogens could possibly increase the predictability of enteropathogen survival. Oligotrophication of the farm environment was also suggested to reduce the overall risk of enteric pathogens in agricultural products (Franz and Van Bruggen, 2008).

Diurnal temperature fluctuations. In natural eco-systems, temperature is never static. Dynamic temperature changes likely have a large influence on the rate of chemical reactions and the autochthonous microbial community as well as on introduced pathogen populations in natural substrates. However, the mechanism of the effects of varying temperatures on survival and adaptation of the pathogens to a new environment compared to static temperatures was still unclear, since almost all survival experiments were carried out under static conditions (Kudva et al., 1998; Himathongkham et al., 1999; Franz et al., 2005). Our results showed that survival of enteropathogens in manure under fluctuating temperatures is generally less than survival under static temperatures at the same mean temperature (Chapter 2). Moreover, the reduction in survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was more pronounced when the amplitude in temperature oscillations was large (7 ºC) compared to that at oscillating temperatures with a smaller amplitude

(4 ºC). We hypothesize that there are two possible explanations for the greater reduction in survival under oscillating than under constant temperatures. A physiological explanation is that an increasing temperature may constitute a greater stress and energy expenditure for a particular microorganism than a decreasing temperature. On the other hand, there is also a mathematical explanation, namely that the difference in survival between oscillating and constant temperatures (with the same mean) is due to the non-linearity of the temperature response, with a relatively greater sensitivity to temperatures higher than the mean compared to temperatures lower than the mean (Scherm and van Bruggen, 1994). This last explanation was corroborated by simulation of pathogen survival under different dynamic temperatures (Chapter 7). No artificial delay in response time was needed to mimic the increased decline under oscillating temperatures compared to constant temperatures.

Oxygen conditions during sampling and plating. Since foods, manure and soil samples are routinely checked for the presence of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium, several standardized detection methods have been developed to screen for both pathogens. Selective plating under aerobic conditions is the most commonly applied procedure. Several selective media have been described (de Boer, 1998): sorbitol MacConkey (SMAC) agar was developed for detection of *E. coli* O157:H7 (March and Ratnam, 1986), while for *Salmonella* serovar Typhimurium common Luria Broth (LB) agar has been used (de Boer, 1998). In general, the sensitivity of these plating methods is assumed to be very high, even though significantly higher numbers of pathogens (especially *E. coli* O157:H7) could be enumerated by direct cell counting methods in comparison to plating methods (Chapter 2). Aerobic incubation of dilution plates at 37-42 ºC is the standard procedure, while the digestive tract of animals is anaerobic and both pathogens are known to be facultative anaerobic bacteria (Minor, 1984; Orskov, 1984). In Chapter 3 we proposed that transfer of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium cells from mostly anaerobic conditions (in natural substrates like manure or slurry) to aerobic conditions (during plating and incubation of Petri dishes) may lead to oxygen stress and lower recovery rates of enteropathogens. In our experiments, more than half of the samplings of *E. coli* O157:H7 resulted in significantly higher numbers of recovered cells when dilution plates were incubated anaerobically in comparison with the common aerobic incubation. *Salmonella* serovar Typhimurium did not show a significant difference in survival in aerobically and anaerobically stored substrates as well as after aerobic and anaerobic incubation of Petri dishes. Therefore, to prevent an underestimation of *E. coli* O157:H7 numbers during surveys and experiments used for risk assessments, Petri dishes should be incubated anaerobically, while for *Salmonella* serovar Typhimurium the common aerobic procedure would be acceptable.

Interaction with autochthonous microbial communities. The microbial community has a large influence on the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and slurry as well as in soil amended with manure. *E. coli* O157:H7 survives longer in autoclaved soils amended with manure than in nonautoclaved soils (Jiang et al., 2002). The same differences in survival are evident for both pathogens in farm-yard manure (Chapter 2). Contrary to the declining populations in fresh manure, populations of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium increase in sterilized manure, indicating that the microbial community has an overriding effect on survival of these enteropathogens. *E. coli* O157:H7 is more sensitive to competition by the native microbial community than *Salmonella* serovar Typhimurium (Chapter 2 and 3). The presence of oxygen has a direct effect on survival of *E. coli* O157:H7 as well as an indirect effect through changes in the autochthonous microbial community composition and the resulting chemical characteristics of the habitat (Chapter 3). Contrary to the response of the individual pathogen species to environmental conditions, the densities of total cultivable bacteria are generally independent of incubation temperature (Chapter 2) and oxygen presence (Chapter 3). However, shifts in microbial composition with changes in temperature are likely and have been shown for changes in oxygen concentration (Chapter 3). For example, aerobic mineralization of carbon sources led to higher numbers of copiotrophic bacteria and a less complex structure of the microbial community, intensifying the competition between the autochthonous microbial community and enteropathogens, while relatively high concentrations of easily available carbon sources in anaerobic conditions increase the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and slurry (Chapter 3). This is in agreement with the finding that natural substrates with a higher microbial diversity are more resistant to stress and disturbance (Girvan et al., 2005; Brussaard et al., 2007)(Chapter 5) and consequently less susceptible to invasion by human pathogens. In this thesis, no direct correlation was found between the survival of *E. coli* O157:H7 and microbial diversity in manure (Chapter 3) or soil (Chapter 4), although a negative relation between biological diversity and survival of *E. coli* O157:H7 was postulated based on comparisons of soils exposed to differential fumigation treatments (van Elsas et al., 2007). However, intensive fumigation does not only result in a reduction in biodiversity but also in an increase in substrate availability, so that these factors may be confounded.

The role of management. The primary habitat of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium is the generally copiotrophic and anaerobic intestine of warm-blooded animals with an abundant supply of easily degradable compounds (Macfarlane and Macfarlane, 1997). After excretion of cow feces and application of manure or slurry to soil, pathogens have to adapt to mostly oligotrophic secondary habitats where the concentration of available carbon and nitrogen sources is usually

very low (Hattori and Hattori, 1976). Therefore, one of the main factors determining the decline of pathogens in soil is their inability to maintain the population at a low concentration of usable organic nutrients (Klein and Casida Jr, 1967; Jamieson et al., 2002). In Chapter 4 we showed that the survival of the copiotrophic *E. coli* O157:H7 in manure-amended soil is indeed shorter in more oligotrophic conditions. Such conditions can be obtained by addition of organic fertilizers with a relatively high C/N ratio and ligno-cellulose fraction, such as farm-yard manure or compost. An increase in C/N ratio and fiber content of organic fertilizers can be achieved by feeding cattle a diet with a higher fiber content or mixing manure with straw (Franz et al., 2005). Application of such manures to soil results in a relatively slow release of easily available nitrogen and carbon sources. This creates a more oligotrophic system which is a disadvantage for the copiotrophic enteropathogens.

Soils with oligotrophic conditions frequently have a higher microbial diversity (van Bruggen and Semenov, 2000), and are more common at farms under organic management (Mader et al., 2002; van Diepeningen et al., 2006). Application of farm-yard manure (high C/N ratio) and slurry (low C/N ratio) to soil can result in different soil organic carbon and nitrogen content, availability of ammonium and nitrate, as well as soil microbial activity (Chapter 6). Organic fertilizers with low C/N ratio lead to relatively more copiotrophic conditions in soil. Soil amendment with such organic fertilizers can result in higher nutrient losses (Reijs et al., 2007), lower natural suppression of plant pathogens (van Bruggen et al., 2006) and to an increased survival of human pathogens. Indeed, higher densities and longer survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were observed in unplanted soil amended with slurry than in that amended with farm-yard manure (Chapter 6). Although, on average the survival time of *E. coli* O157:H7 was not affected by organic versus conventional management type due to the large variation in agricultural practices within each management type (Chapter 4), a high microbial diversity in soil with a high C/N ratio together with minimal soil disturbance (superficial application of farm-yard manure instead of injection of slurry) can lead to more stable conditions with less irregular fluctuations in soil populations (Chapter 5).

Unification of abiotic and biotic factors. The importance of taking the combined influences of biotic and abiotic characteristics on survival of human pathogens into account has been shown in Chapter 4. Temperature (Chapter 2), oxygen concentration (Chapter 3), readily available substrate (Chapters 4 and 6) and competition with autochthonous communities (Chapters 2 and 3) appear to be major factors determining the persistence of enteropathogens in manure as well as in soil after application of organic fertilizers.

To better understand the observed survival patterns of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and soil, the combined effects of different

environmental factors and of the autochthonous microbial community on the survival of an introduced enteropathogen were studied in a simulation model (Chapter 7). In contrast to previously developed descriptive models, the populations of the pathogen (*E. coli* O157:H7) and the competing microbial community were characterized by parameters with clear physiological meaning (such as maximum relative growth and death rates, yield coefficients, recyclable biomass fraction, and intra- and inter-specific competition) in the model COLIWAVE. Separation of specific relative growth rate into relative growth and death rates in relation to substrate concentration, temperature and oxygen content resulted in accurate simulation of the observed oscillatory behavior of a human pathogen in natural substrates (Chapter 7). The oscillations were attained by the interaction between *E. coli* O157:H7 with available substrate content and were not induced by external factors, such as temperature or oxygen changes.

Limitations of the research

Genetically modified strains of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were used in all experiments to be able to monitor their survival more easily and prevent accidental infections of the researchers. *E. coli* O157:H7 contained a green fluorescent protein gene (*gfp*) and ampicillin resistance, while the Shiga-like toxins ($Stx1^-$ and $Stx2^-$) were deleted (Fratamico et al., 1997). *Salmonella* serovar Typhimurium MAE 119 (ΔagfD101 *saw*) carried resistance to kanamycin and gentamycin and contained the *gfp* gene (Römling et al., 2000). The genetic modifications apparently do not affect the intrinsic characteristics of the strain, as no significant behavior differences were observed between *gfp*-transformed strains and parent strains (Fratamico et al., 1997; Römling et al., 2000). Therefore, the use of these strains was supposed to be appropriate for our experiments. However, some uncertainties may remain about the use of genetically modified strains, as it is uncertain to which extent knocked-out genes like virulence and toxin production genes may contribute to survival in manure and soil.

Moreover, only controlled lab experiments could be carried out due to the restriction of GMO usage under field conditions. In these controlled experiments, the initial concentration of the pathogens was around 10^7 CFU gdw^{-1} of manure. Although in natural conditions the density of enteropathogens is usually around 10^4 - 10^5 CFU gdw^{-1}, in some cases the density in contaminated fresh manure can reach 10^7 CFU gdw^{-1} (Fukushima and Seki, 2004). Thus, our initial concentration represented the worst case scenario. Lower initial densities would have resulted in shorter time periods for monitoring enteropathogen persistence in manure and soil. The estimated limit of detection is approximately 10^2 CFU gdw^{-1}. Thus, enteropathogens may still have resided in manure or soil even when they were not detected. Moreover, populations less than 10^2 CFU gdw^{-1} may behave differently, since discrete allocation in space becomes very important at those low densities. Another limitation of the research is

that relatively small soil units were used, mostly without plants, which are not representative of field conditions (Hekman et al., 1994). Only in the experiments described in Chapter 6, we included plants to mimic a rooted soil profile with different organic amendments, so that a realistic assessment could be obtained of the effects of the amendments and water on percolation and survival of the pathogens under more natural conditions.

Implications for practice

With the advent of intensive farming, animal feeding strategies and manure treatment and use changed fundamentally. For example, low fiber diets, including maize silage, are now favored over high fiber diets with mature grasses and straw, and raw manure and slurry are frequently applied instead of aged or composted manure. These modern practices may have contributed to the recent increase in enteritidis outbreaks. Since these outbreaks, composting of manure for at least 90 days became mandatory in the USA before it could be applied to organically farmed cropland (U.S. Department of Agriculture, 2000). In other parts of the world, including Europe, raw manure can still be applied. However, several management options to reduce the risk of vegetable contamination can be proposed based on our research.

Survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure decreases at increasing temperatures as well as at increasing temperature fluctuations (Chapter 2). Both pathogens survive anaerobic conditions in manure or slurry for significantly longer periods in comparison with aerobic conditions (Chapter 3). Turning of a manure heap, as simulated by the COLIWAVE model, reduces the estimated survival time of *E. coli* O157:H7 more than leaving the heap unturned, due to the alternating aerobic and anaerobic conditions in the turned heap, while temperatures are reduced only briefly during turning of the heap. Similarly, Fremaux et al. (2007) observed reduced survival in turned compared to unturned heaps, while the average temperatures did not differ significantly (Fremaux et al., 2007). Kudva et al. (1998) attributed the fast decline in pathogen populations in turned heaps to accelerated drying of manure (Kudva et al., 1998). However, aerobic incubation of manure at a constant moisture content also reduces survival of *E. coli* O157:H7 compared to anaerobic incubation (Chapter 3), so that drying of manure may play only a minor role. Simulated survival of the pathogen is longest on the surface of an unturned manure heap, because the temperature remains relatively low there (Chapter 7). Fremaux et al. (2007) also observed a longer survival at the surface of a manure heap than in the interior, which was partially attributed to a lower pH inside the heap due to organic acid formation (Fremaux et al., 2007). In this thesis a lower pH was also obtained after anaerobic than after aerobic incubation (Chapter 3). Thus, manure and slurry stored at low temperatures and/or in anaerobic conditions should not be distributed onto fields where there is a possibility of contamination of vegetables with human pathogens.

In the Netherlands, injection of slurry is mandatory to reduce ammonia emission (Reijs et al., 2004), and this is practiced both on grasslands and arable fields (including vegetable fields). In Chapter 6, we showed that application of slurry may lead to a significantly higher risk of ground water contamination with *E. coli* O157:H7 and *Salmonella* serovar Typhimurium compared to application of solid manure. The average survival time of *E. coli* O157:H7 at all depths in soil columns was almost twice as high after application of slurry than of manure. When farm-yard manure was applied, neither pathogen was detected at 40 cm depth within a couple of days, even though the manure application was followed by simulated irrigation. Furthermore, the densities of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were significantly higher in the rhizosphere of lettuce plants than in bulk soil after application of slurry, while this was not the case when farm-yard manure was added. Finally, farm-yard manure is preferred over slurry, because manure can create more oligotrophic soil conditions and reduce the survival of the human pathogens due to the lower concentrations of easily available nutrients (Chapter 4 and 5). These findings argue for surface application of farm-yard manure rather than injection of slurry in vegetable fields to reduce the risk of human pathogens associated with vegetable produce.

Implications for future research

Substrates like manure, slurry and soil amended with organic fertilizers play an important role in the introduction of enteropathogens in the vegetable production chain (Natvig et al., 2002). The effects of several abiotic factors (e.g. temperature, oxygen concentration and DOC content) on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in these substrates have been studied in this thesis, but the effects of potentially toxic compounds naturally produced in manure and soil, such as NH_3, N_2O and organic acids, on survival of these pathogens have been insufficiently addressed. NH_3 is mainly released from the surface of manure or slurry at a high pH, while N_2O and organic acids are produced under anaerobic conditions at a low pH (Saggar et al., 2004). These compounds have a very broad toxicity, and could influence pathogen survival negatively, but this has not been studied for naturally occurring NH_3, N_2O or organic acids in manure or slurry. Moreover, very little is known about the combined influence of multiple biotic and abiotic factors on enteropathogen survival, such as the chemical and microbial composition in manure or soil (Guan & Holley, 2003).

E. coli O157:H7 and *Salmonella* serovar Typhimurium interact with the microbial community at all stages of their life cycle, namely in the intestinal tract of animals, in manure and soil amended with manure and, finally, on/in plants. The relations between changes in the autochthonous microbial community and survival of enteropathogen populations were studied in this thesis (Chapter 3). However, it is still unclear to what extent different functional groups of microorganisms might be

important for survival of the pathogens. Some microorganisms could be direct antagonists of the pathogens by occupying the same ecological niches or by producing suppressive compounds. On the other hand, cellulose and lignin degrading organisms may provide *E. coli* O157:H7 and *Salmonella* serovar Typhimurium with less complex carbon sources and enhance their survival. Frequent analysis of the microbial community structure over time by DGGE may reveal significant shifts in the microbial community which could be related to survival of enteropathogens. Sequencing of DNA bands that are unique for substrates that promote a fast decline in enteropathogens may lead to the identification of antagonistic species, which could then be used as probiotics to reduce fecal shedding of *E. coli* O157:H7 by cows (Brashears et al., 2003; Tabe et al., 2008).

Soil management also influences the microbial community and structure of soil organic matter (van Diepeningen et al., 2006; Fließbach et al., 2007) and can therefore have a significant effect on spread and survival of enteropathogens. However, the influence of farming practices on pathogen spread has not been investigated in detail. For example, the role of irrigation in the spread of enteropathogens and the possible contamination of leaf surfaces have been insufficiently investigated (Franz et al., 2008). In addition, it is still unknown if enteropathogens multiply in the rhizo- or endosphere, so that plants could possibly serve as a secondary reservoir.

Finally, the COLIWAVE model can be improved by replacing the constant background flux of substrate (BGF) with an organic matter decomposition model such as BACWAVE-WEB (Zelenev et al., 2006). Separation of pathogenic and autochthonous microbial populations into active and dormant cells as well as division of the autochthonous microbial community over copiotrophic and oligotrophic bacteria (including cellulolytic bacteria) might lead to a more realistic simulation of substrate concentrations and competition for substrate. To enable prediction of movement of enteric pathogens through the soil profile into the groundwater, the COLIWAVE model could be integrated into hydrological models, which simulate transport processes of water and nutrients in soil (e.g. the SWAP model) (van Dam and Feddes, 2000). The data presented in chapter 6 could be used for calibration or validation of such a model.

Conclusions

Temperature and oxygen availability are important external variables affecting survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and soil. Incubation of manure at fluctuating temperatures results in lower survival of these pathogens compared to constant temperatures with the same mean. It may therefore be difficult to accurately predict survival of these pathogens if only effects of static conditions are used for risk assessment. The indigenous microbial communities and availability of substrate are even more important characteristics determining the

survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and manure amended soils. However, there is no clear relation between microbial diversity and the survival of *E. coli* O157:H7 in soil. Thus, the main factors affecting the survival and spread of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium are the quality of organic fertilizers and soil in terms of the extent of eutrophication. The extent of eutrophication is generally less at organic than at conventional farms, indicating that organic management may be less risky in terms of survival and spread of enteric pathogens. Storage and application of manure are often also different at organic compared to conventional farms, as solid and composted manure is more frequently used at organic farms. Application of solid manure results in less survival of these pathogens compared to injection of slurry. Thus, also with respect to manure use organic farms may be less risky than conventional farms where slurry is used. Various management practices were explored with the (semi-) mechanistic simulation model COLIWAVE. This model provides a new approach to investigating dynamic changes of invasive microorganisms in natural substrates such as manure or manure amended soil as affected by various environmental factors (temperature, oxygen concentration and substrate content) and interactions with the autochthonous microbial community. The results presented in this thesis can be used for risk assessment of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in dairy farming systems and will help to identify and evaluate potential control strategies to minimize the chance of pathogen spread in the vegetable production chain.

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Summary

Application of organic amendments such as farm-yard manure or slurry to soil is increasing world wide. The main reason for application of organic fertilizers is the enhancement of soil fertility, since utilization of farm-yard manure and slurry is the most economic and practical option for improving soil quality as well as providing an additional source of nutrients for growing plants. On the negative side, organic fertilizers may contain pathogenic organisms, in particular enteropathogenic bacteria such as *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Substrates like manure and slurry have become a major concern with respect to food safety, since these substrates play an important role in the introduction of enteropathogens in the food chain and have been recently related to a significant increase in outbreaks of enteric diseases. Moreover, many outbreaks have been associated with the consumption of raw produce (like fruits and vegetables), presumably contaminated from animal manure, water, or human handling. The effects of manure quality, substrate availability, microbial competition, temperature, and oxygen concentration on growth and survival of *E. coli* O157:H7 and *S. enterica* serovar Typhimurium had not been documented extensively. Therefore, the main goals of this thesis research were to determine the effects of these factors on multiplication and survival of these enteropathogens in the primary plant production chain and to develop a simulation model for prediction of enteropathogen survival in manure and soil amended with manure under different management scenarios.

In **Chapter 2** effects of temperature fluctuations on survival of the enteropathogens *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were investigated in small microcosms. Four average temperatures (7, 16, 23 and 33 °C) and daily oscillations with three amplitudes (0, ±4, ±7 °C) were used for the experiments. Farm-yard manure was inoculated with these enteropathogens at 10^7 CFU/g dry weight (dw). Manure samples were collected immediately after inoculation, and after one and two or three weeks for *E. coli* O157:H7 or *Salmonella* serovar Typhimurium, respectively. Growth and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were fitted to a modified logistic model. Analysis of the estimated parameter values showed that *E. coli* O157:H7 survived for shorter periods of time and was more sensitive to competition by the native microbial community than *Salmonella* serovar Typhimurium. Survival of both pathogens significantly declined with increasing mean temperatures as well as with increasing amplitude in daily temperature fluctuations. The results indicated that responses of enteropathogens to fluctuating temperatures cannot be deduced from temperature relationships determined under constant temperatures.

The influence of aerobic and anaerobic conditions on the survival of the enteropathogens in broth, cattle manure or slurry was described in **Chapter 3**. Broth, cattle manure or slurry were inoculated with *E. coli* O157:H7 and *Salmonella* serovar

Typhimurium at 10^7 CFU/gdw. Survival data was fitted to the Weibull model. The survival curves in aerobic conditions generally showed a concave curvature, while the curvature was convex in anaerobic conditions. Based on estimated survival times, *E. coli* O157:H7 survived significantly longer under anaerobic than under aerobic conditions. Survival of *E. coli* O157:H7 ranged from approx. 2 weeks for aerobic manure and slurry to more than six months for anaerobic manure. On average, in 56.3% of the samplings (90% for aerobically and 40.1% for anaerobically stored manure and slurry), the number of recovered *E. coli* O157:H7 cells by anaerobic incubation of Petri plates was significantly ($p < 0.05$) higher in comparison with aerobic incubation. On the other hand, survival of *Salmonella* serovar Typhimurium was not different between aerobic and anaerobic storage of LB broth or manure as well as between aerobic and anaerobic incubation of Petri dishes. The importance of changes in chemical composition and autochthonous microbial communities was also shown for the survival of *E. coli* O157:H7 in different oxygen conditions.

To identify risk factors for *E. coli* O157:H7 persistence in soil, survival of this human pathogen was studied for a set of 36 Dutch arable manure-amended soils (18 pairs of neighboring organic and conventional soils). Various biotic and abiotic characteristics of the soils amended with manure were measured **(Chapter 4)**. The Weibull model was used to fit the survival data. The estimated *E. coli* O157:H7 survival times ranged from 54 to 105 days. No differences were observed between organic and conventional soils as well as between sandy and loamy soils. The variation in survival time of *E. coli* O157:H7 was best explained by the level of dissolved organic carbon per unit biomass carbon. Positive correlations were observed between survival times and dissolved organic carbon and nitrogen content. Moreover, survival of *E. coli* O157:H7 increased with a field history of low-quality manure (artificial fertilizer and slurry) compared to high-quality manure applications (farmyard manure and compost). Based on these results, we concluded that *E. coli* O157:H7 decline faster in more oligotrophic soil conditions, which can be achieved by the use of organic fertilizers with a relatively high C/N ratio and consequently a relatively low rate of nutrient release.

The objective of **Chapter 5** was to describe the irregularity of *E. coli* O157:H7 survival in manure-amended soils by measuring the temporal variation around the decline curves, and relate this to soil characteristics. Cow manure inoculated with *E. coli* O157:H7 was mixed with 18 pairs of organic and conventional soils (10% of manure, kg/kg). For 4 of these soil pairs, also 3 different manure densities (5%, 10% and 20%) were compared. All soil-manure mixtures were incubated for two months, and population densities of *E. coli* O157:H7 were quantified weekly. De-trending of survival data was done by modified logistic regression. The residual values were used to assess variation in the changes of *E. coli* O157:H7 population by performing the Approximate Entropy (ApEn) procedure. On average, the irregularity of *E. coli*

O157:H7 populations around the decline curve was greater in conventional than in organic and in loamy than in sandy soils. Multiple regression analysis of irregularity of *E. coli* O157:H7 survival on various soil characteristics showed a positive relation with the ratio of copiotrophic/oligotrophic bacteria, suggesting greater instability at higher available substrate concentrations. Incremental rates of manure application significantly increased the instability for conventional soils only. Estimates of temporal variation of enteropathogen populations by the ApEn procedure can increase the accuracy of predicted survival times and may form an important indication for soil health.

The effect of water application on percolation and survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in soils amended with cattle manure and slurry was investigated for different soil depths in **Chapter 6**. Four treatments were chosen for the first set of experiments: 1) addition of inoculated farm-yard manure on the soil surface; 2) mixing of inoculated farm-yard manure with the top 10 cm of soil; 3) addition of inoculated slurry on the soil surface and 4) injection of inoculated slurry into the top 10 cm of the soil. Homogeneity of water distribution in the soil profile was confirmed by a non-destructive nuclear magnetic resonance (NMR) method. Survival data were fitted to a modified logistic model, and estimated survival times were compared among the four treatments. In the second set of experiments, pathogen-inoculated farm-yard manure or slurry was applied to soil columns with one-month old lettuce plants. More pathogen cells percolated to greater depths after slurry than after manure application. Survival of *E. coli* O157:H7 was significantly longer in soil with slurry than in that with manure, while survival times of *Salmonella* serovar Typhimurium was equally long in soils with manure as in those with slurry. The densities of the pathogens were not different in the rhizosphere compared to the bulk soil treated with manure, while the densities were higher in the rhizosphere than in bulk soil after slurry application. Our results suggest that surface application of manure may decrease the risk of contamination of ground water and lettuce roots compared to injection of slurry.

In addition to the controlled experiments on survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in manure and manure-amended soil, a simulation model for survival of *E. coli* O157:H7 was developed and described in **Chapter 7.** The main objective was to investigate the relative effects of temperature, oxygen concentration and substrate content on the irregular behavior and survival of *E. coli* O157:H7 in manure and manure amended soil. The variations in *E. coli* O157:H7 populations around the decline curves were simulated as oscillations in these populations by the interactions of relative growth and death rates with readily available substrate content. The overall decline in *E. coli* O157:H7 was primarily determined by competition with autochthonous copiotrophic bacteria simulated by an inter-specific competition term according to Lotka-Volterra. The model contains a logistic and

exponential relation of relative growth and death rates, respectively, of *E. coli* O157:H7 and copiotrophic bacteria with temperature, resulting in optimum curves for net growth rates similar to the curves reported in the literature. The model was used to perform sensitivity analysis and to evaluate various manure and soil management scenarios in terms of survival of *E. coli* O157:H7. The relative effects of changes in temperature on simulated survival time of *E. coli* O157:H7 were more pronounced than changes in oxygen condition. Similar to the observed survival times in Chapter 2, survival of the pathogen was reduced more under oscillating temperatures than under constant temperatures with the same mean. Testing manure storage scenarios with realistic values of input variables revealed that manure stored in a heap that was turned every week resulted in almost 70% reduction of *E. coli* O157:H7 survival compared to unturned manure. At the surface of a heap with unturned manure, simulated survival time was the longest (2.4 times longer than inside the same heap). This simulation model, named COLIWAVE, provides a new approach to investigating dynamic changes of invasive microorganisms in natural substrates such as manure or manure amended soil.

Finally, a discussion of the results of all previous chapters is presented in **Chapter 8**. In this chapter major contributions and limitations of the performed research and recommendations for future research are discussed. The autochthonous microbial communities and availability of substrate were the main characteristics of the manure and manure amended soils determining the survival of *E. coli* O157:H7 in these habitats. The main factors affecting the survival and spread of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were the quality of organic fertilizers and soil in terms of the extent of eutrophication and the handling and application methods of these fertilizers. The extent of eutrophication was generally less at organic farms, indicating that organic management may be less risky in terms of survival and spread of enteric pathogens in those production systems. Finally, the model COLIWAVE provides a new approach to investigating dynamic changes of invasive microorganisms in natural substrates as affected by various environmental factors and interactions with the autochthonous microbial community. The results presented in this thesis can be used for risk assessment of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in dairy farming systems and will help to identify and evaluate potential control strategies to minimize the chance of pathogen spread in the vegetable production chain.

Резюме

Внесение отходов животного происхождения в почву распространенная в мире стратегия их утилизации со значимыми природоохранными и агрономическими эффектами (**Глава 1**). Почвенная утилизация твердых и жидких видов навоза не только способствует улучшению качества среды обитания человека, но и наиболее экономичный и практичный способ повышения плодородия почвы, поскольку эти органические остатки являются источником элементов минерального питания, необходимых для растущих растений. Однако внесение в почву свежих животноводческих отходов может иметь также отрицательные последствия из-за опасности присутствия в их массе патогенных организмов, в частности таких энтеропатогенных бактерий, как *Escherichia coli* O157:H7 и *Salmonella* serovar Typhimurium. В этом случае животноводческие отходы становятся потенциальным источником внедрения в пищевую цепь энтеропатогенов с последующей высокой вероятностью возникновения эпидемий среди населения. Известно, что случаи эпидемий тесно связаны с потреблением в сыром виде свежих фруктов и овощей, которые были загрязнены остатками навоза и водой. Поэтому гигиеническая чистота и безопасность продукции является ключевым критерием применения животноводческих отходов в качестве органического удобрения, а идентификация факторов, минимизирующих распространение патогенов, их размножение и выживание на стадии производства растительной продукции, важная предпосылка снижения угроз здоровью людей.

В **Главе 2** данной работы показано действие температурных флуктуаций на выживаемость энтеропатогенов *Escherichia coli* O157:H7 и *Salmonella* serovar Typhimurium. В условиях микрокосма испытывались четыре градации температуры (7, 16, 23 и 33 °C) при трех амплитудах дневных колебаний (0, ±4, ±7 °C). Навоз крупного рогатого скота (КРС) был инокулирован энтеропатогенами из расчета 10^7 КОЕ/г сухого вещества. Образцы навоза с *E. coli* O157:H7 отбирались сразу же после инокуляции, через одну и через две недели, в варианте с *Salmonella* serovar Typhimurium – соответственно после инокуляции, через две и три недели. Количественные показатели роста и выживания инокулятов были описаны модифицированной логистической моделью. Анализ оценочных параметров показал, что *E. coli* O157:H7 отличалась более коротким периодом выживания и меньшей конкурентоспособностью с автохтонным сообществом, чем *Salmonella* serovar Typhimurium. Выживаемость обоих патогенов достоверно снижалась не только при увеличении средней температуры, но и при увеличении амплитуды внутрисуточных колебаний температуры. Эти данные свидетельствуют, что характер отклика энтеропатогенов на температурные флуктуации не может быть установлен по зависимостям, полученным при постоянном значении температуры.

Влияние аэробных и анаэробных условий на выживание энтеропатогенов в питательной среде, в твердом и жидком навозе КРС описано в **Главе 3**. Испытываемые субстраты были инокулированы *E. coli* O157:H7 и *Salmonella* serovar Typhimurium из расчета 10^7 КОЕ/ г сухого вещества. Кривые выживания энтеропатогенов, описанные моделью Вейбулла, имели вогнутый вид при аэробных условиях, а при анаэробных - выпуклый. Жизнеспособность *E. coli* O157:H7 была достоверно продолжительней при анаэробных условиях по сравнению с аэробными, варьируя от двух недель (аэробное хранение твердого и жидкого навоза) до шести месяцев (анаэробное хранение). Число жизнеспособных КОЕ *E. coli* O157:H7 при анаэробной инкубации чашек Петри с образцами, отобранными из анаэробного и аэробного навоза, было достоверно ($p < 0.05$) выше, чем при аэробной инкубации. В тоже время, не было достоверных различий по выживанию *Salmonella* serovar Typhimurium между аэробными и анаэробными вариантами хранения питательной среды или навоза, так же как и различий по жизнеспособности клеток в условиях аэробной или анаэробной инкубации посеянных чашек Петри. Подчеркивается, что жизнеспособность *E. coli* O157:H7 при разной обеспеченности кислородом во многом зависит от сопутствующих изменений химических характеристик субстрата и состава автохтонного микробного сообщества.

Для идентификации факторов, контролирующих устойчивость *E. coli* O157:H7 в почвенной среде, исследовано выживание этого вида патогена в зависимости от совокупности физических, химических и биологических свойств 36 разноудобренных почв Голландии (**Глава 4**). Массив исследуемых образцов включал в себя 18 пар почв с органической или традиционной системой удобрения. Кривая выживания *E. coli* O157:H7, описанная моделью Вейбулла, имела отчетливый вогнутый изгиб, демонстрирующий наличие устойчивости к стрессовым факторам. Период жизнестойкости исследуемого патогена составлял от 54 до 105 суток. Не установлено существенных различий по жизнеспособности *E. coli* O157:H7 между почвами с органической и традиционной системой удобрения, песчаным или суглинистым гранулометрическим составом. Вариабельность периода жизнеспособности организмов достаточно хорошо объяснялась отношением содержаний в почвах растворимого Сорг к С биомассы. Время выживания патогенов положительно коррелировало с содержанием в почвах растворимого Сорг и общего азота. Результаты исследований позволяют предположить, что применение минеральных удобрений или жидкого навоза способствует удлинению периода жизнеспособности *E. coli* O157:H7 в почве по сравнению с подстилочным навозом или компостом. Быстрое снижение количества КОЕ *E. coli* O157:H7 при систематическом внесении органических удобрений с относительно широким отношением C/N обусловлено олиготрофикацией питательного режима почвы,

которая создается благодаря постепенному высвобождению доступных микроорганизмов элементов питания в умеренных количествах.

Глава 5 посвящена характеристике выживаемости *E. coli* O157:H7 в почвах с органическими удобрениями с позиций стабильности популяции, оценивая степень варьирования жизнеспособности в зависимости от свойств почв. Инокулированный *E. coli* O157:H7 навоз КРС смешивали с образцами 18 пар почв с органической и традиционной системой удобрения в количестве 10% от массы почвы. В четырех парах почв дополнительно сравнивали три нормы навоза (5%, 10% and 20% от массы почвы). В смешанных образцах почв с навозом еженедельно на протяжении двух месяцев определяли численность популяции *E. coli* O157:H7. Исключение из массива данных тренда, выполненное с помощью модифицированной логистической модели, позволило проанализировать краткосрочные изменения жизнеспособности популяций. Оценку регулярности изменений популяций *E. coli* O157:H7 производили методом приближенной энтропии. Установлено, что снижение КОЕ *E. coli* O157:H7 было более беспорядочным в почвах суглинистого состава и с традиционной системой земледелия, чем в песчаных почвах и с органическим удобрением. Судя по данным множественной регрессии неравномерность выживания на почвах с разными свойствами достоверно коррелировала с соотношением копиотрофных/олиготрофных бактерий, подтверждая более отчетливую нестабильность популяции при высоком содержании доступного субстрата. Внесение возрастающих доз навоза достоверно изменяло флуктуабельность снижения жизнеспособности *E. coli* O157:H7 только в почве с традиционной системой земледелия. Полученные результаты свидетельствуют, что оценка поведения в почве энтеропатогенных популяций методом приближенной энтропии повышает точность предсказания времени их выживания в почве и может использоваться в качестве одного из индикаторов здоровья почвы.

Жизнеспособность *E. coli* O157:H7 и *Salmonella* serovar Typhimurium, просочившихся с поливной водой из удобренного навозом слоя почвы, рассмотрена в **главе 6**. В первой серии опыта исследовали четыре варианта: 1) внесение подстилочного навоза на поверхность почвы; 2) смешивание инокулированного патогенами подстилочного навоза со слоем 0-10см; 3) поверхностное внесение инокулированного жидкого навоза; 4) инжектирование инокулированного жидкого навоза на глубину 10 см. Равномерность распределения воды в почвенном профиле проверялась методом ЯМР без нарушения сложения почвы. Данные КОЕ энтеропатогенов описывались логистической моделью, вычисляя время их выживания. Во второй серии опыта инокулированный патогенами подстилочный и жидкий навоз вносили в сосуды с одномесячными растениями салата. Установлено, что при внесении жидкого

навоза клетки патогенов просачивалось с водой на более значительную глубину, чем при примененении подстилочного навоза. Жизнеспособность *E. coli* O157:H7 была достоверно продолжительней в почве с жидким навозом, тогда как для *Salmonella* serovar Typhimurium одинаково высокой при обоих видах навоза. Если при внесении подстилочного навоза не было различий по плотности КОЕ патогенов между ризосферой и неризосферной частью почвы, то при применении жидкого навоза в ризосфере обнаруживалось более высокая их плотность, чем в остальном объеме почвы. Полученные результаты показывают, что поверхностное внесение навоза может существенно уменьшить вероятность загрязнения грунтовых вод и корней салата патогенами по сравнению с распространенной технологией внутрипочвенного размещения жидкого навоза.

На основании результатов выполненных экспериментов была разработана математическая модель (**Глава 7**). Цель данной модели предсказывать поведение и жизнеспособность *E. coli* O157:H7 в свежих животноводческих отходах и удобренной навозом почве при разных условиях температуры, концентрации кислорода и содержания субстрата. Флуктуации популяций имитировались зависимостями между относительной скоростью роста/отмирания бактерий и содержанием легкодоступного субстрата, поскольку снижение *E. coli* O157:H7 определялось в первую очередь конкуренцией с автохтонными копиотрофными бактериями, подчиняясь модели межвидовой конкуренции Лотки-Вольтерра. Модель включает логистическую и экспоненциальную связь относительных скоростей роста и отмирания соответственно *E. coli* O157:H7 и копиотрофных бактерий с температурой, следствием чего кривые нетто-роста оказались сходными с известными из литературы. Модель адаптирована для анализа чувствительности *E. coli* O157:H7 к разным внешним условиям и прогноза выживания этого патогена в животноводческих отходах и в почве. Из модели следует, что время жизнеспособности *E. coli* O157:H7 в большей мере зависит от изменений температуры, чем от изменения условий аэрированности. Еженедельное перемешивание хранящего в отвале навоза показало почти 70% снижение жизнеспособности *E. coli* O157:H7 по сравнению с неперемешанным, что соответствовало фактическим результатам. В тоже время, предсказанное моделью время жизнеспособности патогенна на поверхности отвала навоза было в 2.4 раза продолжительнее, чем внутри отвала. Эти и другие результаты имитационной модели свидетельствуют о перспективности ее использования при исследовании динамических изменений потенциально опасных для здоровья людей микроорганизмов в таких природных субстратах как животноводческие отходы и унавоженные почвы.

В **главе 8** дано краткое изложение результатов предыдущих разделов с особым акцентом на их новизну и значимость для практики и исследований в этом направлении.

Samenvatting

Er is een wereldwijde toename in het gebruik van organische meststoffen zoals mest en drijfmest. De belangrijkste reden voor de toepassing van deze organische meststoffen is verhoging van de bodemvruchtbaarheid, omdat het gebruik van vaste mest en drijfmest de meest economische en praktische wijze is om de bodemkwaliteit te verbeteren en omdat het een aanvullende bron van voedingsstoffen is voor het gewas. Een negatief aspect is dat organische meststoffen pathogene microorganismen kunnen bevatten, met name enteropathogene bacteriën zoals *Escherichia coli* O157:H7 en *Salmonella enterica* serovar Typhimurium. Vanuit het oogpunt van voedselveiligheid baart het gebruik van mest en drijfmest zorgen omdat deze meststoffen een belangrijke rol kunnen spelen bij de introductie van enteropathogene bacteriën in de voedselketen. Recentelijk zijn diverse uitbraken van voedselvergiftiging geassocieerd met de consumptie van verse tuinbouwproducten (zoals groenten en fruit), die waarschijnlijk waren besmet door contact met dierlijke mest of water, of ten gevolge van menselijke handelingen. Hoewel er al veel bekend is over de overleving van *E. coli* O157:H7 en *S. enterica* serovar Typhimurium in mest en grond, zijn de effecten van mestkwaliteit, beschikbaarheid van substraat, competitie met andere microorganismen, temperatuur en zuurstofgehalte op de groei en overleving van deze pathogenen nog niet uitvoerig onderzocht. De belangrijkste doestellingen van het onderzoek beschreven in dit proefschrift zijn daarom: het vaststellen van de effecten van deze factoren op de vermeerdering en overleving van deze enteropathogene bacteriën in de primaire productieketen van verse groenten en het ontwerpen van een simulatiemodel voor het voorspellen van hun overleving in mest en bemeste grond onder verschillende beheersingsregimes.

In **hoofdstuk 2** zijn de effecten van temperatuurschommelingen op overleving van de *E. coli* O157:H7 en *Salmonella* serovar Typhimurium onderzocht in kleine microcosmos systemen. Effecten van vier gemiddelde temperatuurwaarden (7, 16, 23 en 33^{o}C) met dagelijkse schommelingen van 0, ±4 of ±7^{o}C zijn in deze experimenten vergeleken. Hiervoor werd stalmest in afzonderlijke porties geënt met 10^{7} kolonievormende eenheden (KVE)/g drooggewicht (gdw) van één van beide enteropathogenen. Monsters werden onmiddellijk na enting genomen, na één week en na twee of drie weken voor experimenten met respectievelijk *E. coli* O157:H7 of *Salmonella* serovar Typhimurium. Groei en overleving van *E. coli* O157:H7 en *Salmonella* serovar Typhimurium werden beschreven met behulp van een aangepast logistisch model. Uit de verkregen parameterwaarden bleek dat de levensduur van *E. coli* O157:H7 korter was en dat dit pathogeen meer gevoelig was voor competitie met de inheemse microbiële levensgemeenschap in mest dan *Salmonella* serovar Typhimurium. Overleving van beide pathogenen was significant korter bij een hogere gemiddelde temperatuur, maar ook bij een toenemende amplitude in dagelijkse temperatuurschommelingen. Hieruit blijkt dat de overleving van enteropathogenen onder wisselende temperatuursomstandigheden niet direct afgeleid kan worden uit de overleving bij constante temperaturen.

De invloed van zuurstofrijke (aerobe) en zuurstofarme (anaerobe) omstandigheden op de overleving van enteropathogenen in een kweekmedium, koemest of drijfmest werd beschreven in **hoofdstuk 3**. Een kweekmedium, koemest en drijfmest werden geënt met 10^7 KVE van *E. coli* O157:H7 of *Salmonella* serovar Typhimurium per g drooggewicht. De aantallen overlevende KVE over een periode van 50 dagen werden beschreven door een Weibull model. De overlevingscurven van beide pathogene bacteriën waren hol van vorm voor aerobe omstandigheden, en bol voor anaerobe omstandigheden. Dit verschil in algemene vorm van de curven duidt op een aanvankelijk betere overleving onder anaerobe omstandigheden. De berekende overlevingsduur voor *E. coli* O157:H7 was significant langer onder anaerobe dan onder aerobe omstandigheden. Overleving van *E. coli* O157:H7 varieerde van ongeveer 2 weken in aeroob bewaarde mest en drijfmest tot meer dan 6 maanden in anaeroob bewaarde vaste mest. Gemiddeld werden in 56.3% percent van de mest en drijfmestmonsters waaruit *E. coli* O157:H7 werd geïsoleerd significant grotere aantallen colonies ($P < 0.05$) gevonden in anaeroob bewaarde dan in aeroob bewaarde petrischalen (90% voor de mestmonsters die eerst zelf aeroob bewaard waren, en 40.1% voor de monsters die eerst anaeroob bewaard waren). Aan de andere kant was er geen verschil in de overleving van *Salmonella* serovar Typhimurium in kweekmedium of mest die aeroob of anaeroob werd bewaard, noch in overleving op agar medium in petrischalen die aeroob en anaeroob geïncubeerd werden. De overleving van *E. coli* O157:H7 werd mede beïnvloed door veranderingen in chemische samenstelling en de inheemse microbiële levensgemeenschap in mest en drijfmest bewaard bij verschillende zuurstofgehaltes.

Om het risico van de persistentie van *E. coli* O157:H7 in grond vast te stellen, werd de overleving van deze ziekteverwekker bestudeerd in monsters van 36 verschillende landbouwgronden in Nederland (18 aanpalende biologisch en gangbaar bewerkte akkers) na menging met besmette mest. Verscheidene biotische en abiotische eigenschappen van deze grond-mest mengsels werden bepaald (**hoofdstuk 4**). De afnemende aantallen KVE over een periode van 60 dagen werden weer beschreven door een Weibull model. De berekende *E. coli* O157:H7 overlevingstijd varieerde tussen de 54 en 105 dagen. Er werd geen verschil aangetroffen tussen biologische en gangbare bewerking van grond en tussen zanderige en leemachtige bodemtypes. De variatie in overlevingstijd van *E. coli* O157:H7 werd het best verklaard door de hoeveelheid opgeloste organische koolstof per eenheid koolstof vastgelegd in biomassa. Positieve correlaties werden waargenomen tussen overlevingstijd en het gehalte aan opgeloste koolstof en stikstof. Verder was de overleving van *E. coli* O157:H7 hoger in grond afkomstig uit velden waar mest van een lage kwaliteit (kunstmest en drijfmest), dan waar mest van een hoge kwaliteit (stalmest en compost) was toegediend. Gebaseerd op deze resultaten concluderen wij dat de *E. coli* O157:H7 populatie sneller afneemt wanneer bodemomstandigheden meer oligotroof zijn. Deze omstandigheden kunnen worden bereikt door het toedienen van organische meststoffen met een relatief hoge C/N verhouding, waaruit gemakkelijk opneembare voedingsstoffen langzamer vrij komen.

De doelstelling van **hoofdstuk 5** was om de stabiliteit in afnemende *E. coli* O157:H7 populaties in grond gemengd met mest vast te stellen door de variaties in overleving te meten en te relateren aan bodemeigenschappen. Koeienmest gemengd met *E. coli* O157:H7 werd toegediend aan 18 grondmonsters (10 % mest, kg/kg) afkomstig van paren van biologisch en gangbaar bewerkte akkers. In vier van deze bodemparen werd het effect van toediening van drie verschillende mesthoeveelheden (5 %, 10 % en 20 %) op variatie in overleving gemeten. De *E. coli* O157:H7 dichtheid werd wekelijks vastgesteld in alle bodem-mest mengsels gedurende een periode van twee maanden. Via een aangepaste logistische regressie werden alle trendmatigheden uit de overlevingsgegevens verwijderd. De resterende waarden werden gebruikt om de variatie in *E. coli* O157:H7 populaties te schatten met behulp van de 'Approximate Entropy' (ApEn) procedure. Gemiddeld was de onregelmatigheid (instabiliteit) in *E. coli* O157:H7 populaties rond de afname curve groter in gangbaar dan in biologisch bewerkte grond en groter in leemachtige dan in zanderige bodems. Multipele regressie analyse van de onregelmatigheid van *E. coli* O157:H7 op verschillende bodemeigenschappen toonde een positief verband van deze onregelmatigheid met de verhouding tussen copiotrofe en oligotrofe bacteriën. Dit zou een aanwijzing kunnen zijn voor een grotere instabiliteit bij een hogere hoeveelheid beschikbaar substraat in de bodem. Naarmate de hoeveelheid toegediende mest toenam was er grotere instabiliteit, vooral bij gangbaar bewerkte grond. Door de onregelmatigheid in het populatieverloop van enteropathogenen in grond te schatten met behulp van de ApEn procedure kan de overlevingsduur met grotere nauwkeurigheid worden voorspeld. De relatie tussen de mate van stabiliteit van een populatie en oligotrofe omstandigheden suggereert dat ApEn metingen een indicatie kunnen vormen voor de mate van bodemgezondheid.

Het effect van watertoediening op het doordringen van *E. coli* O157:H7 en *Salmonella* serovar Typhimurium naar diepere grondlagen (percolatie) en overleving op verschillende dieptes na menging van de toplaag met koemest of drijfmest werd onderzocht in **hoofdstuk 6**. Vier behandelingen met één van beide pathogenen werden toegepast in een eerste serie experimenten: 1) toediening van geënte stalmest op het grondoppervlak; 2) vermenging van de bovenste 10 cm grond met geënte stalmest; 3) aanbrengen van geënte drijfmest op het grondoppervlak en 4) injectie van geënte drijfmest in de bovenste 10 cm grond. Met behulp van een niet-destructieve 'nuclear magnetic resonance'(NMR) methode werd een homogene verdeling van water in het bodemprofiel vastgesteld. Overleving van beide pathogenen werden beschreven met behulp van een aangepast logistisch model. De daaruit geschatte overlevingsduur werd vergeleken voor de vier behandelingen. In een tweede serie experimenten, werden geënte stalmest en drijfmest monsters gemengd met grond in een kolom waarin één-maand oude slaplanten groeiden. Cellen van beide pathogenen drongen dieper door in grond waaraan drijfmest was toegediend dan in grond met vaste mest. Ook was de overlevingsduur van *E. coli* O157:H7 significant hoger in grond met drijfmest dan in die met vaste mest, terwijl de overlevingsduur van *Salmonella* serovar Typhimurium gelijk was in met drijfmest en vaste mest behandelde grond. De dichtheden van beide

pathogenen in de rhizosfeer verschillenden niet van die in de bulkgrond na toediening van vaste mest, terwijl de populaties in de rhizosfeer significant hoger waren dan die in de bulkgrond na toediening van drijfmest. Onze resultaten suggereren dat het risico van besmetting van slawortels in de bodem en van grondwaterbesmetting geringer is wanneer stalmest oppervlakkig wordt uitgereden dan wanneer drijfmest wordt geïnjecteerd.

Naast de experimenten over de overlevingsduur van *E. coli* O157:H7 en *Salmonella* serovar Typhimurium in mest en met mest behandelde grond werd een simulatiemodel ontwikkeld dat de overleving van *E. coli* O157:H7 beschrijft (**hoofdstuk 7**). De belangrijkste doelstelling was het vaststellen van het effect van temperatuur en zuurstof- en substraatgehalte op de populatiedynamiek en overlevingsduur van *E. coli* O157:H7 in mest en met mest behandelde grond. De dynamiek van *E. coli* O157:H7 populaties werd beschreven in een simulatiemodel in de vorm van oscillaties die het resultaat waren van tegenovergestelde relaties tussen the relatieve groei en sterfte snelheden en het beschikbare substraatgehalte. De uiteindelijke afname in *E. coli* O157:H7 werd verondersteld het gevolg te zijn van competitie met inheemse copiotrofe bacteriën en werd gesimuleerd door een Lotka-Volterra interspecifieke competitieterm. De relaties tussen de relatieve groei- en sterftesnelheden en temperatuur worden beschreven door een logistisch, respectievelijk een negatief exponentieel verband voor zowel *E. coli* O157:H7 als inheemse copiotrofe bacteriën. Deze constructie resulteert in optimum curves voor netto groeisnelheden die overeenkomen met eerder gepubliceerde gegevens uit de literatuur. Het model werd gebruikt om gevoeligheidsanalyses uit te voeren en om het effect van verschillende mest- en bodembeheersingsscenario's te evalueren op overlevingsduur van *E. coli* O157:H7. Verandering van temperatuur had een relatief groter effect dan aan- of afwezigheid van zuurstof op de gesimuleerde overlevingsduur van *E. coli* O157:H7. De gesimuleerde afnamesnelheden van *E. coli* O157:H7 populaties waren groter bij wisselende dan bij constante temperatuurwaarden met dezelfde gemiddelden, zoals eerder beschreven voor de afnamesnelheden geobserveerd in hoofdstuk 2. Uit simulaties met verschillende mestopslagscenario's, waarbij realistische waarden voor de inputvariabelen werden gebruikt, bleek dat het wekelijks keren van een mesthoop leidde tot een reductie in overlevingsduur van *E. coli* O157:H7 van bijna 70% ten opzichte van een niet-gekeerde hoop. De gesimuleerde overlevingsduur was het langst aan het oppervlak van een niet-gekeerde mesthoop (2,4 keer langer dan binnenin dezelfde hoop). Dit simulatiemodel, COLIWAVE genoemd, vormt een nieuwe benadering om dynamische veranderingen van geïntroduceerde microorganismen in natuurlijke substraten zoals mest en grond gemengd met mest te beschrijven.

Ten slotte wordt een discussie over de resultaten in alle voorafgaande hoofdstukken gepresenteerd in **hoofdstuk 8**. In dit hoofdstuk worden belangrijke bijdragen en beperkingen van het uitgevoerde onderzoek met aanbevelingen voor toekomstig onderzoek bediscussieerd. De inheemse microbiële levensgemeenschap en beschikbare hoeveelheid substraat waren de belangrijkste factoren van mest en met mest gemengde gronden die de overlevingsduur van *E. coli* O157:H7 in deze

natuurlijke leefomgevingen bepaalden. De belangrijkste factoren die gerelateerd waren aan de overleving en verspreiding van *E. coli* O157:H7 en *Salmonella* serovar Typhimurium waren de kwaliteit van organische meststoffen en van de grond met betrekking tot de mate van eutrofiëring, alsmede de behandeling en toedieningwijze van deze meststoffen. De mate van eutrofiëring was over het algemeen lager op biologische boerderijen, wat aangeeft dat biologische beheersingsmaatregelen mogelijk minder risicovol zijn in termen van overleving en verspreiding van enteropathogenen in deze productiesystemen. Tenslotte vormt het COLIWAVE model een nieuwe benadering om dynamische veranderingen in geïntroduceerde microorganismen in natuurlijke substraten onder verschillende omstandigheden te simuleren door hun interactie met inheemse microbiële levensgemeenschappen. De resultaten gepresenteerd in dit proefschrift kunnen worden gebruikt voor risico-inschattingen van *E. coli* O157:H7 en *Salmonella* serovar Typhimurium in melkveehouderijen en zullen bijdragen aan de identificatie en evaluatie van mogelijk belangrijke beheersingsstrategieën om de kans van verspreiding van pathogenen in de plantaardige voedselproductieketen te minimaliseren.

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Alexander V. Semenov
Wageningen, October 2008.

Curriculum vitae

Alexander V. Semenov was born on 4th of June, 1982 in Puschino, Moscow region, Russia. After high school, he studied Soil Biology in the Soil Science department at Moscow State University. After preparation of his MSc thesis "Production and consumption of methane by a grey forest soil under different management activities", he received his MSc diploma in 2004. In 2002 and 2003, he was invited to the Biological Farming Systems Group of Wageningen University for several months to study the effect of organic management on the suppressiveness of soils to *Gaeumannomyces graminis* var. *tritici* and the survival and biological control activity of its antagonist, *Pseudomonas fluorescens*. In 2004, he was admitted into the PhD program of the graduate school Production Ecology and Resource Conservation at Wageningen University to carry out a research project entitled "Ecology and simulation modelling of *E. coli* O157:H7 and *Salmonella* Typhimurium survival in cattle manure, slurry and soil". Currently, he is working as a Postdoctoral Researcher in the Microbial Ecology department of the Centre for Ecological and Evolutionary Studies at the State University of Groningen.

Publication list

Semenov, A.V., van Overbeek, L., and van Bruggen, A.H.C. (2008) Influence of manure and slurry applications on *Escherichia coli* O157:H7 and *Salmonella* serovar Typhimurium survival and their translocation through a soil column. *Applied and Environmental Microbiology, submitted.*

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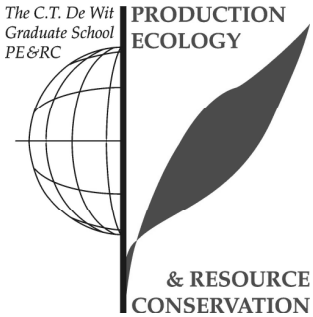
Educational certificate of the Graduate School PE&RC

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises a minimum total of 32 ECTS (= 22 weeks of activities)



Review of Literature (5.6 ECTS)

- Survival and spread of enteropathogens in manure-soil-plant chains as affected by environmental, microbial and abiotic factors (2004)

Writing of Project Proposal (7 ECTS)

- Survival and spread of enteropathogens in manure-soil-plant chains as affected by environmental, microbial and abiotic factors (2005)

Laboratory Training and Working Visits (2.0 ECTS)

- Identification of pathogens with Taqman PCR; PRI (2005)

Post-Graduate Courses (3.4 ECTS)

- Advanced statistics; PE&RC (2005)
- Science of Organic Production: from Ecology to Socio-Economics; (2005)

Deficiency, Refresh, Brush-up Courses (2.8 ECTS)

- System Analysis, Simulation and Systems Management; BFS, PPS (2004)

Competence Strengthening / Skills Courses (1.6 ECTS)

- Working with EndNote; PE&RC (2004)
- English course; CENTRA (2005)

Discussion Groups / Local Seminars and Other Scientific Meetings (7 ECTS)

- Biological Farming Systems Group: temporal agricultural production systems (2004-2005)
- Bacteriology meeting (2004-2008)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.5 ECTS)

- PE&RC annual meeting “Biological Disasters”(2005)
- PE&RC weekend (2005)

- NWO/WUR workshop Human pathogens in the vegetable production chain (oral presentation) (2006)

International Symposia, Workshops and Conferences (7 ECTS)

- 90th Annual Meeting of the Ecological Society of America, Montreal, Canada (2006)
- 6th International symposium on Vertoxin producing *Escherichia coli* infections, Melbourne, Australia (2007)
- The International Association for Food Protection, Columbus, Ohio, USA (2008)

Courses in Which the PhD Candidate Has Worked as a Teacher:

- System Analysis, Simulation and Systems Management; 2006 BFS 24 days
- System Analysis, Simulation and Systems Management; 2007 BFS 24 days
- System Analysis, Simulation and Systems Management; 2008 BFS 24 days

Supervision of MSc student(s)

- Survival and spread of enteropathogens in manure-soil-plant chains as affected by environmental, microbial and abiotic factors 60 days