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

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Abstract

The effects of four average temperatures (7, 16, 23 and 33 °C) and daily oscillations with three amplitudes (0, ±4, ±7 °C) on the survival of the enteropathogens *Escherichia coli* O157:H7 and *Salmonella* serovar Typhimurium were investigated in small microcosms. Manure was inoculated with a green fluorescent protein transformed strain of either pathogen at 10^7 cells g^{-1} dry weight. Samples were collected immediately after inoculation, and 1 and 2 weeks after inoculation for *E. coli* O157:H7, and immediately and after 2 and 3 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 on a global level (Flint *et al*., 2005; Rangel *et al*., 2005). The annual cost of illness in the US due to *Escherichia 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 the consumption of uncooked vegetables and fruits, presumably contaminated by animal manure, water, or human handling (Beuchat, 2002; Rangel *et al*., 2005). *Escherichia coli* O157:H7 is very dangerous due to its low infective dose (as few as 10 cells) and high pathogenicity (Tilden *et al*., 1996). *Salmonella* serovar Typhimurium is less pathogenic but widespread 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 & Vågsholm, 2005; Fossler *et al*., 2005; Hussein & Sakuma, 2005). Thus, preventing their accumulation in cattle and reducing their survival in feces are important avenues for reducing the risk of contamination of plant products and reducing the risk of food-borne diseases (Franz *et al*., 2005). The 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 °C (Nicholson *et al*., 2005). With the advent of intensive farming, manure treatment and use changed fundamentally from the application of raw manure and slurry in many parts of the world. Due to the recent increase in enteritis outbreaks, composting of manure became mandatory in the USA before it could be applied to cropland; however, this is only mandatory for organic farms (US Department of Agriculture, 2000). In other parts of the world, raw manure is still applied.

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Various manure characteristics, such as chemical composition (Franz *et al.*, 2005) and 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, 2004; Kudva *et al.*, 1998; Himathongkham *et al.*, 1999) lead to differences in the 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. *Escherichia coli* O157:H7 survived from several days at 37 °C to several months at 4 °C 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 does *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 °C (Kudva *et al.*, 1998; Himathongkham *et al.*, 1999), although the optimal temperature for these enteropathogens under laboratory conditions in broth is about 37 °C (Minor, 1984; Orskov, 1984).

The microbial community also has a great impact on the survival of enteric pathogens. *Escherichia coli* O157:H7 survived significantly longer in manure-amended autoclaved soil than in manure-amended nonautoclaved soil at 15 °C (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 the 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 & 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 the 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 nonsterilized manure and to investigate the effect of competition and/or antagonism from the autochthonous microbial community.

Materials and methods

Bacteria

Escherichia 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*) (pGFP cDNA vector) and ampicillin resistance, whereas 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 Römling *et al.* (1998, 2000). This strain carried resistance to kanamycin and gentamicin 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^{-1}, dry weight)] and stored (50–60 °C at 20 cm depth) for nearly 1 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 2 weeks. To obtain sterilized manure for

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

Manure	pH	Dry matter ($g\,kg^{-1}$)	N-NH_4 ($g\,kg^{-1}$)	Total N ($g\,kg^{-1}$)	Total C ($g\,kg^{-1}$)	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

some experiments, a plastic, hermetically sealed jar with manure was gamma-irradiated with 39.6 kGy from Co_{60} (Isotron, Ede, the Netherlands) for 1 day. The sterile condition of the manure was checked by dilution-plating of *c.* 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 ($g\,v^{-1}$). 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).

Inoculation of manure

Bacterial inocula were grown in Erlenmeyer flasks containing 150 mL fresh Luria–Bertani broth, with 50 $\mu g\,mL^{-1}$ ampicillin for *E. coli* O157:H7 and 50 $\mu g\,mL^{-1}$ kanamycin (both from Sigma-Aldrich Chemie Gmbh, Germany) for *Salmonella* 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 suspension was determined using the spectrophotometer, where the OD 0.7 at 630 nm in 1 mL cuvet would equal *c.* 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 ($g\,dw^{-1}$) 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 (diameter 52 mm). The Petri plates were closed by PetriSEAL (DiversifiedBiotech), 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 (diameter 86 mm) and closed by PetriSEAL.

Setup of experiments

Four different experiments were carried out in sterilized and nonsterilized manure to establish the influence of static vs. fluctuating temperatures on the 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 three amplitudes, ±4 and ±7 °C, with 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 two amplitudes, ±7 and 0 °C as control, respectively). Selection of the temperature treatments for the experiments was based on seasonal temperature variations in soil (Stoller & 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 1 and 2 weeks for *E. coli* O157:H7, or after 2 and 3 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) to prevent a sudden temperature change at the start of each experiment. Each cell contained one Petri dish with inoculated manure. There were two control plates with noninoculated manure per experiment, maintained at constant temperatures (7, 16, 23 or 33 °C).

Sampling procedure of manure

Samples of manure, *c.* 0.5 g, were put in preweighed 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). Tenfold 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 $\mu g\,mL^{-1}$) for *E. coli* O157:H7 and on Luria–Bertani agar with kanamycin (50 $\mu g\,mL^{-1}$) for enumeration of *Salmonella* serovar Typhimurium. After adding *c.* 20 sterile glass beads per Petri dish, stacks of several plates were repeatedly shifted in different directions to allow the glass

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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 CFUs 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. Each sample in 50 μL 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 epifluorescent 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 g dw^{-1}.

Statistical analysis

First, the number of colonies for each Petri plate was calculated to CFU g dw^{-1} and SDs were calculated for every temperature treatment. ANOVA tests were done for CFUs and direct cell counts per gram of dry manure after 2 weeks, to assess the influence of temperature and its fluctuations on the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium using the General Linear Models (GLM) procedure of the SAS program (SAS Institute Inc., Cary, NC). To describe the decline in CFUs and direct counts over time in nonsterilized 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 g dw^{-1} at time t (days), a is the upper asymptote (CFU g dw^{-1}), c is a parameter for the shoulder (days), and m is a slope parameter for the rate of change (days^{-1}). This model was selected because we previously showed excellent fits of data on the decline of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in similar cattle manure, where six 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. The significance and fit of the estimated decline rates were assessed by the F value of the nonlinear regression and the nonlinear coefficient of determination (pseudo-r^2) for each curve, respectively. The growth and decline of *Salmonella* serovar Typhimurium in sterilized manure were 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 g dw^{-1} at time t (days), N_0 is initial log CFU g dw^{-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 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 g dw^{-1} of untreated manure. Pathogen populations significantly declined during the 2-week incubation period in fresh manure at each of the temperature treatments (Fig. 1a). At a constant temperature of 7 °C, CFUs decreased slightly but significantly ($P < 0.05$) to 7.44 ± 0.10 log CFU g dw^{-1} after 2 weeks. At 7 ± 4 and 7 ± 7 °C, there were slightly fewer CFUs remaining after 14 days than at a constant temperature of 7 °C, but the differences between oscillating and constant temperatures were not significant. Treatments with a mean temperature of 16 °C resulted in a more rapid decrease in CFUs than those at 7 °C, the final densities ranging from 6.96 ± 0.05 log CFU g dw^{-1} for the static variant to 6.85 ± 0.12 and 6.63 ± 0.12 log CFU g dw^{-1} for 16 ± 4 and 16 ± 7 °C, respectively. The final density at 16 ± 7 °C 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 °C ($P < 0.001$), where log CFUs decreased to 6.09 ± 0.05 g dw^{-1} after 2 weeks. The effects of temperature oscillations were more pronounced at a mean temperature of 23 °C than at lower mean temperatures ($P < 0.05$). At 23 ± 4 and 23 ± 7 °C, the final densities dropped to 5.67 ± 0.27 and 5.43 ± 0.16 log CFU g dw^{-1}, respectively. Finally, at 33 °C, *E. coli* O157:H7 declined so quickly that after 1 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 g dw^{-1}. After 2 weeks at an average temperature of 7 °C, there was a slight drop to 7.84 ± 0.11 regardless of temperature oscillation (at 7 ± 7 °C the final density was 7.79 ± 0.09 log CFU g dw^{-1}). Manure samples exposed to 16 and 23 °C showed significant ($P < 0.05$) growth in all

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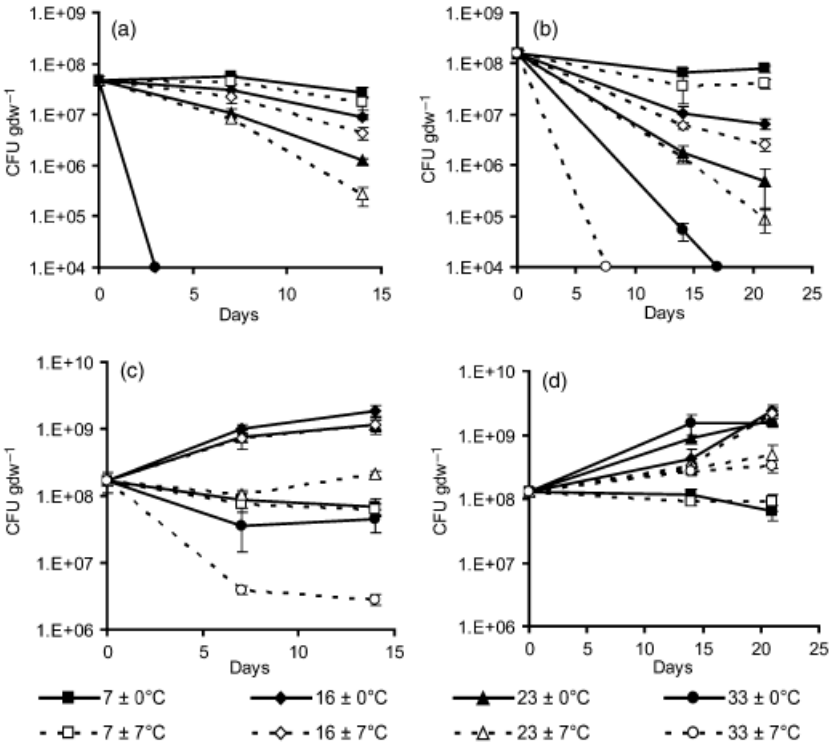

Fig. 1. Survival of *E. coli* O157:H7 (a, c) and *Salmonella* serovar Typhimurium (b, d) at different temperature levels for treatments with static temperatures and with $\pm 7\,^{\circ}C$ oscillations in untreated (a, b) and sterilized (c, d) manure. Data for treatments with $\pm 4\,^{\circ}C$ oscillations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

cases, with final densities reaching 9.27 ± 0.08 and 9.05 ± 0.09 log CFU g dw^{-1} at 16 and $16 \pm 7\,^{\circ}C$, and 9.05 ± 0.12 and 8.32 ± 0.05 log CFU g dw^{-1} at 23 and $23 \pm 7\,^{\circ}C$, respectively. Contrary to the nonsterilized manure, in sterilized manure *E. coli* O157:H7 was found at $33\,^{\circ}C$, although it did not increase initially as it did at 16 and $23\,^{\circ}C$. Oscillating temperatures at $33 \pm 7\,^{\circ}C$ resulted in a decline to 6.46 ± 0.25 log CFU g dw^{-1} compared to 7.65 ± 0.20 log CFU g dw^{-1} at constant $33\,^{\circ}C$. At all mean temperatures except $7\,^{\circ}C$, the 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 $r^2 = 0.96$; $P < 0.001$) $= -6.92 \times 10^{-3} \times \mathrm{MT}$ ($P < 0.001$) $- 3.54 \times 10^{-3} \times \mathrm{AT}$ ($P < 0.05$) (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 $7\,^{\circ}C$, 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 $23\,^{\circ}C$), whereas the declines in population (7 and $33\,^{\circ}C$) 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 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 nonsterilized manure (at 8.19 ± 0.06 log CFU g dw^{-1}), the density of this enteropathogen had decreased slightly to 7.82 ± 0.08 log CFU g dw^{-1} at a constant temperature of $7\,^{\circ}C$. Temperature oscillations led to a more rapid decline down to 7.69 ± 0.14 and 7.48 ± 0.26 log CFU g dw^{-1} at 7 ± 4 and $7 \pm 7\,^{\circ}C$, respectively, although only the CFU density of the last temperature treatment was significantly different from the other treatments with an average temperature of $7\,^{\circ}C$ ($P < 0.05$). At a constant temperature of $16\,^{\circ}C$, survival was significantly less than at $7\,^{\circ}C$, down to 7.00 ± 0.19 log CFU g dw^{-1}. Under

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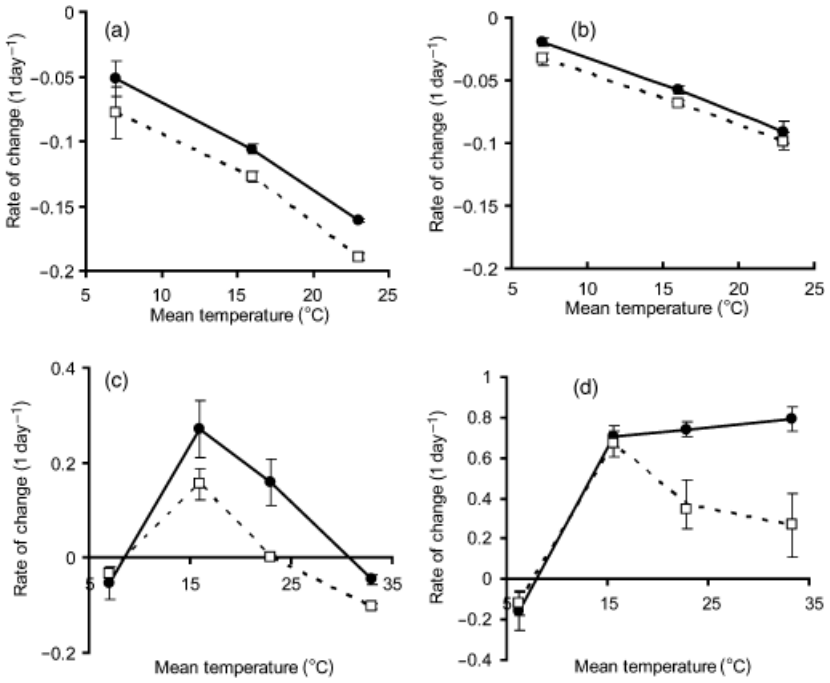

Fig. 2. Relative rates of change (day^{-1}) for *E. coli* O157:H7 (a, c) and *Salmonella* serovar Typhimurium (b, d) at three or four mean temperatures (7, 16, 23 and 33 °C) (□) and without oscillation (●) in untreated (a, b) and sterilized (c, d) manure. Vertical bars represent standard deviations.

oscillating temperatures survival was even less, down to 6.80 ± 0.18 and 6.77 ± 0.07 log CFU g dw^{-1} at 16 ± 4 and 16 ± 7 °C, respectively. CFUs decreased to 6.23 ± 0.16 log CFU g dw^{-1} within 2 weeks at 23 °C. 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 g dw^{-1} at 23 ± 4 and 23 ± 7 °C, respectively. In contrast to *E. coli* O157:H7, *Salmonella* serovar Typhimurium was detected by plate counting after 2 weeks at 33 °C at a density of 4.64 ± 0.42 log CFU g dw^{-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 °C.

In sterilized manure samples, there was a significant increase in CFUs at all temperature treatments after 2 weeks, except at 7 °C (Fig. 1d). The densities at 7 °C were 7.96 ± 0.06 and 8.07 ± 0.09 log CFU g dw^{-1} after 2 weeks of oscillating and constant temperatures, respectively, compared to the initial density of 8.10 ± 0.70 log CFU g dw^{-1}. At 16 and 16 ± 7 °C, populations increased to 8.59 ± 0.17 and 8.51 ± 0.03 log CFU g dw^{-1}. Similar population densities were obtained after incubation for 2 weeks at 23 and 23 ± 7 °C, namely 8.95 ± 0.04 and 8.47 ± 0.08 log CFU g dw^{-1}, respectively. Finally, in contrast to *E. coli* O157:H7, *Salmonella* serovar Typhimurium showed the greatest increase at 33 and 33 ± 7 °C to a density of 9.16 ± 0.17 and 8.40 ± 0.15 log CFU g dw^{-1}, respectively, after 2 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 $r^2 = 0.98$; $P < 0.001$) $= 1.42 \times 10^{-2}$ ($P < 0.001$) $- 4.56 \times 10^{-3} \times$ MT ($P < 0.001$) $- 2.31 \times 10^{-3} \times$ AT ($P < 0.01$) (Fig. 2b).

Similar to the 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 °C (Fig. 2d).

Influence of static and oscillating temperatures on direct counts of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in nonsterilized manure

At constant temperatures, the cell densities of *E. coli* O157:H7 counted with the epifluorescent microscope decreased slightly from 7.75 ± 0.20 to 7.64 ± 0.14 log cells g dw^{-1} at 7 °C, to 7.30 ± 0.19 log cells g dw^{-1} at 16 °C, and to 7.29 ± 0.24 log cells g dw^{-1} at 23 °C after 2 weeks. In two of nine manure samples stored at 33 °C, 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 no significant effects of temperature

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oscillations, probably because of the 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 g dw^{-1} did not change significantly at 7 °C, but changed to 7.34 ± 0.16, 7.18 ± 0.11, and 6.97 ± 0.13 log cells g dw^{-1} at 16, 23, and 33 °C, respectively. Mean temperature as well as time of sampling had significant effects ($P < 0.05$) on the density of *Salmonella* serovar Typhimurium cells, but the effects of temperature amplitudes were not significant.

Influence of static and oscillating temperatures on densities of total cultivable bacteria in nonsterilized manure

Densities of total bacterial CFUs on LB plates without antibiotics were similar after 1, 2 and 3 weeks of incubation at all different treatments of oscillating and static temperatures, averaging 8.56 ± 0.08 log CFU g dw^{-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 g dw^{-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 < 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 from < 7 days for *E. coli* O157:H7 at 33 °C to 159 days (theoretical estimation, viz. the number of days needed to reach 1 log CFU g dw^{-1}) at 7 °C (Table 2). For *Salmonella* serovar Typhimurium, the survival period, predicted from the model, ranged from 227 days at 7 °C to < 21 days at 33 °C (Table 3). Oscillating temperatures decreased survival more than expected from the same mean temperatures under static temperature conditions, especially at higher mean temperatures. After 2 weeks of incubation of inoculated untreated manure at oscillating temperatures (± 7 °C), 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 the 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 epifluorescent microscope, but the sensitivity of this method was much lower than that of dilution plating.

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

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

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

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

Discussion

Similar to other studies on the survival of enteropathogens in manure (Wang *et al.*, 1996; Kudva *et al.*, 1998; Himathongkham *et al.*, 1999; Wang *et al.*, 2004), the survival of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium in untreated manure decreased at increasing temperatures. The 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 show 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

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populations, it is well known that oscillating temperatures with the same mean as static temperatures influence the 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 $23 \pm 5\,^{\circ}C$ with period of 1 day) compared to constant temperatures (Economos & 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 modeling (Scherm & 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 & van Bruggen, 1994). The survival of *Fusarium oxysporum* and of *Sclerotium rolfsii* was also significantly different under fluctuating temperature and relative humidity compared 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, more noticeably at higher than at lower mean temperatures (Fig. 1). In addition, the reduction in survival of either pathogen was more pronounced at an amplitude of $7\,^{\circ}C$ than of $4\,^{\circ}C$.

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 the survival of these enteropathogens. *Escherichia coli* O157:H7 seemed to be more sensitive to competition by the native microbial community than did *Salmonella* serovar Typhimurium, as the temperature effects in untreated manure relative to those in sterilized manure were greater 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–$23\,^{\circ}C$) and high ($33\,^{\circ}C$) 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 ($37\,^{\circ}C$) (A.V. Semenov, unpublished data). This suggests that the 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 nonlinearity 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 & 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 ecosystems, 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 farmyard manure under dynamic temperatures. Although in natural conditions the density of enteropathogens is usually around 10^4–10^5 CFU g dw^{-1}, in some cases the density in contaminated fresh manure can reach 10^7 CFU g dw^{-1} (Fukushima & Seki, 2004). Our initial concentration of *E. coli* O157:H7 and *Salmonella* serovar Typhimurium was 10^7 CFU g dw^{-1}, representing the worst case scenario. The presence of the *gfp* plasmid does not affect the intrinsic characteristics of the strains, 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

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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. *Escherichia 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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