In interpretation of these data, experimental techniques and definitions are important. Some experimenters measured the absolute initial number or concentration of spores (by total spore counting, or by an optical method calibrated by total spore counting), and then measured spores that germinated by colony counts after incubation on suitable media. Such measurements will be referred to as "absolute" in what follows. Other experimenters measured both the effective initial number of spores and the number that germinated by techniques that depended entirely on incubation on suitable media, so may have entirely omitted any spores that never germinated under the conditions of the experiments. Such measurements will be referred to as "relative" in what follows.

- Wynne and Harrell (1951) used an uncharacterized strain of *C. perfringens* in a relative method that indicated 98.5% germination rate after a single heat treatment, with subsequent 1.5% further germination after a second heat treatment, the combined effects of two heat treatments being defined as 100%. The exact methodology is not clear, and raw results are not given. This is the only experiment identified that attempted to recover spores that had not germinated after incubation after the initial heat treatment with a subsequent heat treatment. It is thus the closest available match to the expected sequence of events for some RTE foods — an initial cooking step during manufacture, followed by a re-heat during preparation.

- Wynne *et al*. (1954) again used an uncharacterized strain (possibly the same one) of *C. perfringens* in a relative approach to estimate 94% and 100% relative germination rate after a single heat treatment in two experiments. Measurement in this case was of spores, rather than vegetative cells produced by germinated spores, and recovery of spores was generally by incubation for 2 to 3 days with no second heat treatment — vegetative cells instead were destroyed by contact with oxygen. However, one test performed with a second heat treatment could be interpreted (along with the 100% relative germination rate test, in which there were no recovered spores) as showing approximately 0.2% additional germination after a second heat treatment.

- Ahmed and Walker (1971) used changes in optical density to estimate an alteration in spores that correlated with subsequent germination (as measured by colony counting), as a function of time after heat treatment using *C. perfringens* strain S45. It appears that the method used was an absolute one (the calibration methodology used was not described in sufficient detail to make a complete determination). They measured a maximum optical density change corresponding to approximately 47% germination within 25 minutes after heat treatment at 75 °C for 20 minutes, with a smaller maximum change for 80 °C heat treatment. The optical density change increased approximately linearly with time after heat treatment until it saturated, and for lower heat treatment temperatures the optical density change was apparently still progressing at the end of the experiments.

- Tsai and Riemann (1974) measured the activation of five strains (NCTC 8798, S79, 80535, ATCC 3624, and BP6K; the first three strains listed are associated with food poisoning, the last two are classical or well studied, but not associated specifically with foodborne illness) for various time and temperature combinations of heat treatment. The maximum germination

rates, measured with an absolute method (absolute optical count of initial number of spores; colony counts for germinating spores) ranged from 30% to 70%.

- Craven and Blankenship (1985), using strain NCTC 8679 and a relative measurement method, observed maximum activation with heat treatment at 75 °C for any period longer than about 5 minutes, and defined such conditions as giving 100% activation. Addition of lysozyme increased activation to 105%, (significantly higher than without lysozyme) so that relative activation without lysozyme was at most actually 100/105 = 95%. Using an absolute method, the same authors measured an absolute activation (corresponding to 100% relative activation on their scale) of 61 ± 19%.

The experiments described were conducted in laboratory media and water, using heat-resistant and heat-sensitive (or unknown) *C. perfringens*. A study investigating *C. perfringens* germination in meat indicated a very large relative fraction of spores germinating after heat treatment (but not lysozyme treatment), although no quantitative estimates could be derived (Barnes *et al.*, 1963), and only two studies were found that used heat-resistant strains.

3.9.4. Spore germination fractions after heat treatment — η and g_p

The spore germination fractions required in the model could be either relative fractions or absolute fractions, so long as both are well-defined and used consistently (use of relative fractions would be justified if there are spores that do not germinate in meat products under any conditions met in food processing, storage, transport, and preparation). Two fractions are required; the first (symbolized by η above) for initial processing, and the second (g_p in equation (3.2)) for reheating during food preparation. It is likely that these fractions vary with the strain of *C. perfringens*, and with conditions of heat-treatment, neither of which can currently be modeled.

To encompass the range of measurements described above, the varied heat treatments expected, and the variation in *C. perfringens* strains, η is modeled as varying from 5% to 75% (of the initial total number of spores, corresponding to absolute measurements in Section 3.9.3) with a triangular distribution with a mode of 50%. The effect of these assumptions about distribution shape and values is evaluated using a sensitivity analysis.

Only one experiment (Wynne and Harrell, 1951) effectively measured g_p, and that with near-optimum initial heat treatment for the strain tested. In that circumstance it appeared that few spores remained after the initial heat treatment that could be activated by subsequent heating. If the conditions of the original heat treatment are not optimal, however, and any re-heating approaches optimal conditions, it appears likely that a larger fraction than measured by Wynne and Harrell (1951) could be activated by the second heating. The estimate for g_p is thus conditioned on η — it is treated as variable from 0 to $(0.75-\eta)/(1-\eta)$ (the upper limit corresponding to the assumption that there is an upper bound of 75% in the total fraction of spores that might be activated by up to two heat treatments), with a triangular distribution with mode half way between zero and the upper limit. The effect of these assumptions about distribution shape and values is evaluated using a sensitivity analysis.

3.9.5. Spore germination in favorable conditions without heat treatment

The fraction of spores (symbolized by ϕ above) that germinate in favorable conditions (but without heat treatment) is required to interpret the experiments on spices (Section 3.8.3). The following studies were used to estimate the fraction of spores that germinate in favorable conditions.

- Barnes *et al.* (1963) measured 3% apparent germination and growth (relative to recovery after heat activation) of spores prepared by lysozyme treatment of a spore and vegetative cell suspension of *C. perfringens* F2985/50. However, subsequent incubation at 37 °C led to less than 3.5 logs of growth in the following 24 hours in either raw or cooked meat, suggesting a much extended delay period for any viable remaining vegetative cells or germinating spores. In other tests examining the effect of storage temperature, raw beef blocks were inoculated with a suspension of spores and vegetative cells and stored at constant temperature. Barnes *et al.* (1963) indicate a failure of spores to germinate at all temperatures tested. However, these tests could not distinguish between germination and death of spores, and for temperatures below 15 °C have been assumed to correspond to (see Section 3.13.2) to spore death.

- Roberts (1968) observed that culture counts of unheated spore suspensions were 0.13–3.6% of the microscopically determined total spore count for four or five heat-resistant strains (NCTC 8238, 8239, 8798, 8797, and perhaps 9851; the paper is not clear), but 31–46% for two classical strains (NCTC 3181, 8084). However, it was also observed that the spore preparation method, involving inactivation of vegetative cells by oxygen, was not completely effective, so some of the culture count may have been due to surviving vegetative cells.

- Ahmed and Walker (1971), indicated the presence of some microscopically visible germination after storage of spores frozen for 1 or 2 months (temperature not specified).

- Tsai and Riemann (1974) measured recoveries from spore preparations of 4%, 6% and 8% for three food-poisoning associated *C. perfringens* strains that were not heat treated, and 10% and 13% for two classical strains, although it is not clear to what extent the spore preparations were free of vegetative cells. These recoveries are colony counts for germinating spores, but the initial number of spores was apparently measured optically, so these are absolute recoveries.

- Craven and Blankenship (1985), using type A strain NCTC 8679, observed that 4% to 6% (relative to recovery after heat treatment of 75 °C for 20 minutes) of a spore suspension without heat treatment (<1% vegetative cells, stored desiccated) formed colonies on TSC. Addition of lysozyme increased colony counts to about 10% of the spores in this experiment. The absolute recovery corresponding to 100% relative recovery was 61 ± 19%, so the absolute germination rate is approximately 2% to 4%.

As for spore germination with heat treatment, spore germination fractions without heat treatment are expected to vary with strain of *C. perfringens* and with conditions. To encompass the measurements described above, ϕ for Type A, CPE positive strains is modeled as variable with a

triangular distribution ranging from 1% to 10%, with a mode of 5%. A sensitivity analysis is performed on these parameters and distribution shape to determine the effect of this set of assumptions.

3.10. The fraction (f_{vma}, f_{smA}, f_{vsA}, and f_{ssA}) of *C. perfringens* cells that are type A, CPE-positive

C. perfringens food poisoning is caused by *C. perfringens* type A, CPE-positive (see *Hazard Identification*), and is not typically associated with other types of *C. perfringens*. Measurements and estimates of concentrations in foodstuffs (above) have been made without regard to the type of strain, or to toxin production potential. Consequently, it was necessary to estimate the fraction of *C. perfringens* cells and spores that are type A, CPE-positive. As seen below, no data were available to distinguish how such fractions might vary throughout the preparation of foods, nor to distinguish between vegetative cells and spores in raw meat (presumably the measurements in spices were of spores). Thus no data are available to distinguish the fractions identified as f_{vmA} and f_{smA} in equation (3.1), nor to distinguish the fractions identified there as f_{vsA} and f_{ssA}. In the analysis that follows, each pair of fractions is assigned a single value. These fractions represent the probabilities for any *C. perfringens* isolate found in food to be type A, CPE-positive. It is possible that such probabilities vary in systematic ways, perhaps geographically or temporally. However, in this analysis they are treated as independent of the particular serving of RTE or partially cooked food — they are not variable, only uncertain.[24]

3.10.1. Selection of studies measuring prevalence of type A strains, prevalence of CPE-positive strains, or both

Experimental measurements that may allow some inference about the proportion of type A and/or CPE-positive strains are summarized in Table 3.17. The studies by Kokai-Kun *et al*. (1994), Skjelkvale *et al.* (1979) and Rodriguez-Romo *et al*. (1998) measured only the fraction of samples that were positive for the *C. perfringens* enterotoxin gene (*cpe*) rather than those that were type A. Songer and Meer (1996) and Daube *et al.* (1996) measured both genotype and *cpe* status (presence of DNA for the CPE toxin[25]), the former also demonstrating excellent agreement between *cpe* status and CPE toxin production in classically characterized cell lines.

The first four studies listed in Table 3.17 were measurements of isolates from mammalian or food samples, whereas the last (Rodriguez-Romo, 1998) was of isolates from spices. The first four studies were therefore considered most appropriate to use for estimating the prevalence of type A, CPE+ strains in raw meats, while the last was used only for estimation of prevalence in spices.

[24] The analyses described in this section are performed in the workbook CP_typeA.xls included with the risk assessment.

[25] CPE refers to the fully formed *C. perfringens* enterotoxin protein. *cpe* refers to the DNA gene encoding the CPE toxin.

Table 3.17 Proportion of *C. perfringens* environmental isolates that were type A.

Reference	Source	No. Samples	% cpe-positive	% both CP type A and *cpe+*	% of *cpe+* not CP type A	Experimental Method
Songer and Meer, 1996	USA; primarily human and mammal isolates	616	8.1 % (50/616)	7.1 %	12 % (6/50)	PCR analysis
Daube et al., 1996	Belgium; primarily human and mammal isolates	2,659	1.8 %	1.6 %	12.2 % (6/49)	Colony hybridization with DNA probes
Kokai-Kun *et a*l., 1994	Canada and USA; primarily human and mammal isolates	454	3.5 %	3.1%[a]		PCR analysis
Skjelkvale *et al*., 1979	UK and Norway; mammal feces, meats and foods	168 (not associated with outbreaks or infections)	1.2%	1%[a]		Functional enterotoxin assay
Rodriguez-Romo *et al.*, 1998	Spices in Mexico	188	4.3%	3.7%[a]		Dot-blot with DNA probes.

[a] Percent *cpe+ C. perfringens* type A adjusted by the percent of *cpe+* strain not *C. perfringens* type A.

The summary proportions in Table 3.17 may overestimate or underestimate the proportion of *C. perfringens* type A spores capable of causing *C. perfringens* food poisoning for several reasons, including:

1) The studies did not evaluate whether the isolates containing *cpe* actually produce the enterotoxin (CPE). It is therefore possible that some of the isolates were not capable of causing disease (Kokai-Kun *et al*., 1994). This would result in an over estimate of the proportion of *C. perfringens* type A spores capable of causing *C. perfringens* food poisoning.

2) The studies did not distinguish between *C. perfringens* type A cells that harbored *cpe* on a plasmid and those that harbored *cpe* on the chromosome. Cells of the former are thought to cause sporadic gastrointestinal illness that is not related to food poisoning. Therefore, these cells harboring *cpe* on the plasmid most likely did not represent *C. perfringens* spores capable of causing food-borne disease

(Sarker *et al*., 2000). This would result in an over estimate of the proportion of *C. perfringens* type A spores capable of causing *C. perfringens* food poisoning.

3) Isolates were obtained in a non-random fashion, with often unidentified fractions of them from humans, mammals, or food samples associated with intestinal, if not diarrheal, illness. In particular, the Songer and Meer (1996) isolates appear to have been heavily biased to CPE+ strains (at least 44% of the isolates from Pennsylvania were identified as CPE+; the sources were listed as human, human food or unknown, with no statement as to association with human disease). Daube *et al.* (1996) indicated that of 769 samples (providing their 2659 isolates), 76 were associated with diarrhea (37/46 in humans, although clostridial disease was not suspected), 458 with enterotoxemia, and 10 with necrotic enteritis. This could result in either an over or under estimate of the proportion of *C. perfringens* type A spores capable of causing *C. perfringens* food poisoning depending on how representative these studies are of the prevalence of *C. perfringens* type A spores in meats.

4) The proportion of environmental *C. perfringens* isolates that are of type A may not accurately mirror that found in meat products either before or after initial processing. This could result in either an over or under estimate of the proportion of *C. perfringens* type A spores capable of causing *C. perfringens* food poisoning depending on the true prevalence.

Very few isolates were stated to be derived solely from human foods not associated with disease outbreaks. A subset of 45 of the isolates in Daube *et al.* (1996) was identified as coming from 32 samples of human food not associated with human disease episodes; all isolates in this subset were type A and *cpe*-negative. Of 17 isolates from human food identifiable in Songer and Meer (1996), all were type A, and at least one was *cpe*-positive. However, association with or independence of disease was not reported for these isolates. Of 168 isolates from meat carcasses, minced beef, food and feces not associated with disease, and pig feces, 2 were *cpe*-positive, both in pig feces (Skjelkvale *et al.*, 1979).

In view of the non-randomness identified above in the Songer and Meer (1996) isolates, these data were not used. Selected data from Daube *et al*. (1996), Kokai-Kun *et al*. (1994), and Skjelkvale *et al*. (1979) were used: to increase the representativeness of the data from these papers, only isolates associated with cattle, sheep, pigs, fowl, and human food (not associated with food-poisoning outbreaks) were analyzed to estimate the proportion of Type A, CPE-positive cells associated with meat and meat products. For spices, the only data available are from Rodriguez-Romo *et al*. (1998), and these were used.

There are limited other data that may be correlated with the proportion of *C. perfringens* type A present in foods (Table 3.18). These studies estimated the frequency of *C. perfringens* heat resistant strains in raw and processed meats. These data were not used for the reasons stated below:

1) *C. perfringens* strains were not typed.

2) *C. perfringens* strains were not analyzed for *cpe* gene or the CPE toxin.

3) Though heat resistance is correlated with those *C. perfringens* strains that cause *C. perfringens* food poisoning, heat resistance alone does not predict the potential to cause human disease.

4) Changes have occurred in the slaughter and processing conditions during the past 35 years that may have affected the fraction of *C. perfringens* Type A present.

Table 3.18 Proportion of heat resistant *C. perfringens* among food samples.

Reference	Source	Samples	Heat-resistance (HR)	% heat resistant CP spores
Hall and Angelotti, 1965[a]	OH, USA	Raw and processed meats	Spores "resisted heating at 100 °C for 30 min or more."	1.9% (2/108) of positive retail food samples harbored CP HR strains
McKillop, 1959[b]	Scotland, UK	Raw beef, sausage and chicken	Samples "immersed in a bath of boiling water for 15 mins."	3.6% (2/55)
Bauer *et al*., 1981[a]	GA, USA	Pork	Spores surviving heating at 95 °C	6% (2/34) at 30 min 0% (0/34) at 60 min
Hobbs and Wilson, 1959[b]	Imports to UK from 4 unknown countries	Veal, beef, lamb, mutton, pork	Meat sample jars were “steamed for one hour.”	11% (76/722) Boneless 1.5% (3/195) Carcass
Weadon, 1961[b]	UK	Raw meats	Meat sample jars placed in “shallow water bath kept constantly boiling for 1 hr.”	18% (130/714)

a. Authors isolated *C. perfringens* vegetative cells, induced sporulation, then tested for heat resistance.
b. Authors heat exposed samples, and then tested samples for presence of *C. perfringens*.

3.10.2. Analysis of selected studies for the fraction of *C. perfringens* in raw meat and spices that are type A, CPE-positive

To estimate the fraction of *C. perfringens* cells and spores in raw meat and spices that are type A, CPE-positive, selected data from Daube *et al*. (1996), Kokai-Kun *et al*. (1994), and Skjelkvale *et al*. (1979) were used for raw meat; and from Rodriguez-Romo *et al*. (1998) for spices. No data specifically distinguishing spores from vegetative cells were located; the fractions were assumed to be identical.

Daube *et al.* (1996) typed their isolates using gene probes, and similarly identified those isolates that were *cpe*+. In view of the good agreement observed between genotype and phenotype (Songer and Meer, 1996) for all toxins (including CPE), the genotype was assumed in this analysis to correspond to the phenotype (for both the type A/non-type A and CPE/non-CPE dichotomies), although in principle (at least for CPE) the two may be different because *cpe* could be located on a plasmid rather than in the chromosome (Sarker *et al*., 2000). Kokai-Kun *et al*. (1994), Skjelkvale *et al*. (1979) and Rodriguez-Romo *et al*. (1998) provided data only on *cpe*

status. The selected information (the set of all data on isolates associated with cattle, sheep, pigs, fowl, and human food and not associated with food-poisoning outbreaks) is summarized in Table 3.19.

Table 3.19 Summary of selected data analyzed for fraction of *C. perfringens* expected to be type A, CPE+.

Source of data	Type	Number of isolates	
		cpe+	*cpe-*
Daube *et al.* (1996)	Type A	8	1780
	Non-A	4	20
Kokai-Kun *et al.* (1994)	Unknown	5	201
Skjelkvale *et al*. (1979)	Unknown	2	166
Rodriguez-Romo *et al.* (1998)	Unknown	8	180

Preliminary analysis showed that the *cpe*+ fractions in the first three studies are homogeneous, and this was subsequently confirmed by the analysis described below. It was assumed that among the individual cells of *C. perfringens* infecting meat products (either as spores or vegetative cells) there is a fraction A^+ that are type A, *cpe*+, a fraction nA^+ that are non-A, *cpe*+, a fraction A^- that are type A, *cpe*–, and a fraction $nA^-=1-(A^+ + nA^+ + A^-)$ that are non-A, *cpe*–. Similarly, for spices there are corresponding fractions S^+, nS^+, S^-, and $nS^-=1-(S^+ + nS^+ + S^-)$. Then the observations in Table 3.19 are binomial samples, allowing the corresponding loglikelihood for their observation to be written using suitable combinations of these probabilities. The contribution to the loglikelihood from each entry in Table 3.19 can be written as

$$r \ln\left(pN / r \right) \qquad \textbf{(3.11)}$$

where r is the observed count, N is the total number observed in the study, and p is a suitable combination of the probabilities A^+, nA^+, A^-, nA^-, S^+, nS^+, S^-, and nS^- (see Table 3.20).

For spices, there are insufficient data to estimate from measurements what fraction of the isolates are type A or non-A. It was assumed that within each *cpe* category (+ and –), the relative fraction of type A and non-A were the same as for meat and other foods (the first three studies listed). That is, the additional constraints

$$\begin{aligned} nS^+ &= S^+ \, nA^+ / A^+ \\ nS^- &= S^- \, nA^- / A^- \end{aligned} \qquad \textbf{(3.12)}$$

were imposed. It then follows that

$$S^- = \frac{1 - S^+ \left(1 + nA^+ / A^+ \right)}{1 + nA^- / A^-} \qquad \textbf{(3.13)}$$

Table 3.20 Probabilities for each entry in Table 3.19.

Source of data	Type	Probabilities	
		cpe+	*cpe*–
Daube *et al*. (1996)	Type A	A^+	A^-
	Non-A	nA^+	$1-(A^+ + nA^+ + A^-)$
Kokai-Kun *et al.* (1994)	Unknown	$A^+ + nA^+$	$A^- + nA^- = 1-(A^+ + nA^+)$
Skjelkvale *et al*. (1979)	Unknown	$A^+ + nA^+$	$A^- + nA^- = 1-(A^+ + nA^+)$
Rodriguez-Romo *et al.* (1998)	Unknown	$S^+ + nS^+$	$S^- + nS^- = 1 - S^+ + nS^+$

With these assumptions, maximum likelihood estimates for the independent parameters A^+, nA^+, nA^-, and S^+ were obtained (any four parameters can be treated as the independent ones, and the maximum likelihood estimates are just the obvious values obtained as ratios of the values in Table 3.19, but only A^+ and S^+ are of direct interest here). Using the loglikelihood contributions normalized as in Equation (3.11) ensures that the loglikelihood behaves approximately as a χ^2_6 variate, allowing a test for homogeneity between the studies. They are homogeneous by this test (p=0.54).

Uncertainty estimates were obtained by first finding a suitable transform to make the profile likelihoods for A^+ and S^+ approximately normal (see Appendix 3.1 for discussion of such transformations). The transformations selected are

$$u = \left(\frac{A^+}{1-A^+}\right)^{0.4} \quad \text{and} \quad v = \left(\frac{S^+}{1-S^+}\right)^{0.25} \qquad \textbf{(3.14)}$$

which give excellent normal approximations to the profile likelihoods at least out to 4.5 standard deviations. The maximum likelihood estimates for A^+, S^+, u, and v are given in Table 3.21.

Table 3.21 Maximum likelihood estimates for the fractions of cells that are type A, CPE-positive.

A^+	0.00579
S^+	0.0284
u	0.128
v	0.413

Re-writing the likelihood in terms of u and v allowed quadratic approximation of their local joint profile likelihood using an information matrix (estimated by separately and together making increments in u and v equal to about 1.5 times the standard deviations indicated by their individual profile likelihoods, re-optimizing with respect to the nuisance parameters nA^+ and nA^-, and solving the resultant simultaneous quadratic equations for the change in loglikelihood). An estimate of the variance-covariance matrix for u and v was then obtained by inverting the

information matrix. The resultant estimates for standard deviations and the correlation coefficient are given in Table 3.22.

Table 3.22 Standard deviations (main diagonal) and correlation coefficient (off-diagonal) for the uncertainty distribution of *u* and *v*.

	u	*v*
u	0.0156	0.257
v	0.257	0.0417

3.11. The growth of *C. perfringens* and *C. botulinum*

3.11.1. Modeling growth of *C. perfringens* and *C. botulinum* as a function of temperature and time

Modeling of growth for *C. perfringens* is discussed in technical detail in Appendix 3.2. The methods of that appendix are used here. A model for growth of *C. botulinum* is needed to respond to one of the questions to be answered (Section 1.1), and this is formulated in exactly the same way as for *C. perfringens*.

Growth from spores of *C. perfringens* at fixed temperatures after a heat treatment and in suitable surroundings may be characterized by a delay period t_m during which the activated spore converts to a vegetative state and prepares for cell division. The resultant vegetative cell then enters the growth phase in which cell division occurs regularly, causing an exponential increase with time in cell density, until the density of vegetative cells becomes so high that some aspect(s) of the environment becomes unfavorable for further growth (for example, the cells might run out of food, or produce mutually self-inhibitory chemicals). The growth phase is characterized by a doubling time (the time for cell density to double) or a growth rate (the ratio of the rate of increase in cell density to the cell density itself). The growth rate, symbolized by μ and measured in units of inverse time, is used here. Subsequent behavior, after the vegetative cells have reached the stationary phase at high cell density, is of less concern to this risk assessment. Cell densities would generally decline somewhat, and in suitable conditions the vegetative cells might start sporulating. In favorable environments, such as meats, cell densities in the stationary phase may reach 10^8 to 10^{10} cells per gram. Where necessary in this risk assessment, it is assumed that cells remain at the same high density in stationary phase — although in foods, *C. perfringens* at such cell densities generally imparts a definite "off" odor and taste.

As discussed in Appendix 3.2, the delay period t_m and the growth rate μ depend on the history of the spore or vegetative cell's environment. The temperature of the environment has a major effect on both, although it is generally believed that μ at any time depends principally on the

temperature at the same time, whereas t_m depends strongly on temperature history. For constant temperatures, this risk assessment uses a primary growth[26] model of the form

$$C_s(t) = C_0\left(1 - I\left(a+1, at/t_m\right)\right)$$
$$C_v(t) = f\left(t, T, C_0, \mu, t_m, C_m, a\right) \equiv C_m \frac{z(t)}{1+z(t)} \quad \textbf{(3.15)}$$

$$z(t) = \frac{C_0}{C_m} e^{\mu t}\left(\frac{a}{a+\mu t_m}\right)^{a+1} I\left(a+1, t\left(\mu + a/t_m\right)\right) \quad \textbf{(3.16)}$$

where I is the incomplete gamma integral

$$I(\alpha, x) = \frac{1}{\Gamma(\alpha)}\int_0^x w^{\alpha-1}e^{-w}dw \quad \textbf{(3.17)}$$

and the various terms are

- $C_s(t)$ the spore cell density at time t,
- $C_v(t)$ the vegetative cell density at time t,
- f the mathematical function representing the primary model,
- C_0 the initial spore density (cells/gram),
- T the temperature, with $\mu = \mu(T)$ and $t_m = t_m(T)$,
- C_m the maximum density of cells that can be supported, and
- a an additional variance parameter of the model that indicates how variable t_m is between individual spores under similar conditions (the standard deviation of t_m is approximately $t_m/\sqrt{a}$).

The secondary models describe how μ and t_m vary with temperature; both are of Ratkowsky form,[27] the first for μ and the second for $1/t_m$. These curves may be characterized by maximum and minimum temperatures, the location of the maximum of the curve, and the magnitude of the curve at the maximum (see Appendix A3.2.4). The models take the form

$$\mu = \mu(T) = A_m \frac{(1-x)^2\left(1-\exp\left(-\theta_m x\right)\right)}{N_m} \quad \textbf{(3.18)}$$

and

$$1/t_m = 1/t_m(T) = A_t \frac{(1-x)^2\left(1-\exp\left(-\theta_t x\right)\right)}{N_t} \quad \textbf{(3.19)}$$

where

$$x = \frac{T_{\max} - T}{T_{\max} - T_{\min}} \quad \textbf{(3.20)}$$

is a location on the curve and the terms are:

[26] The "primary" model is the fixed temperature model that relates cell density to time. The "secondary" models describe how the parameters of the primary model vary with temperature. The primary model here is "model 3" of Appendix 3.2

[27] The Ratkowsky form is used because that is the form used in the majority of the literature. L. Huang (personal communication 2004) has pointed out that the Ratkowsky shape may be inadequate for modeling the variation of growth rate, particularly at temperatures near T_{max}.

T the temperature,
T_{max} the maximum temperature for growth or progression through the delay period,
T_{min} the minimum temperature for growth or progression through the delay period,
θ, N functions of the location of the maximum of the curve (see Equations (A3.2.33) and (A3.2.34)).

3.11.2. Method of evaluation of growth rates of *C. perfringens* and *C. botulinum*

The primary model (Equations (3.15) and (3.16)) was used to fit measured growth of *C. perfringens* at fixed temperatures. Data on estimated cell densities as a function of time were obtained (personal communications, 2003, with L. Huang, H. Marks, and V.K. Juneja) for the experiments described by Juneja *et al.* (1999) in broth; Juneja *et al.* (2001) in cooked cured beef; Juneja and Marks (2002) in cooked cured chicken; Huang (2003) in cooked ground beef; and Juneja and Marks (1999) for *C. botulinum* in reinforced Clostridial medium (RCM) supplemented with oxyrase enzyme. These experiments were performed with the sterile growth medium initially inoculated with spores that were then activated to germinate with a heat treatment. Growth media were maintained at constant temperatures thereafter, and samples taken (either by sub-sampling liquid media, or the use of multiple small samples of meat media) at appropriate intervals to measure cell counts by plating.

It was assumed in these experiments that what was measured (as CFU/g) was the sum of vegetative cell and remaining spore densities, $C_s(t) + C_v(t)$ in the notation of Equation (3.15), and that the logarithms of the experimentally estimated CFU/g have normal measurement errors[28] with equal standard deviations at all cell densities. For each temperature replicate in each experiment (with multiple temperatures), the values of C_0, μ, and t_m were estimated. For each experiment, the parameters C_m, a, and the common standard deviation for the measurement errors were estimated. The method of estimation used was maximum likelihood — all parameters associated with a given experiment were obtained simultaneously by maximizing the likelihood with respect to all those parameters. The original investigators' censoring of the measurement data was used — where original authors censored whole replicates for microbiological or experimental reasons (*e.g.* suspected overgrowth, bad thermostat) the same censoring was performed. Where replicates were dropped from analysis by the original authors because there were too few data points to support their analysis approach, the same was generally done, unless those data could sustain the current analysis approach. For Juneja *et al*. (2001) the data above the early exponential part of the growth curve were not censored as in that original paper (which used an approximation to the growth curve only valid in the early portion of the curve), since the growth curve used here tracks the growth curve above that region.[29]

This approach allowed evaluation of maximum likelihood estimates for all the parameters for each experiment, except for the variance parameter a. The likelihood function is a very slow function of a, because the experiments are not sensitive to its value — its value affects only the

[28] In this analysis, the measurement error is assumed to measure the deviations (assumed random) from an ideal mathematical form that occur for the time points within each replicate growth curve.

[29] Except for points in two replicates, both at 21.1°C. The last point in the first replicate and the last 3 in the second replicate were censored (as was done by the original authors). The first one dropped 2 logs between 48 and 54 hours, the second 1.94 logs between 39 and 44 hours and stayed down at 48 and 53 hours.

shape of the growth curve between the initial constant spore density (during the delay period) and the period of exponential growth. A value of $a = 100$ was selected (corresponding to an assumption of about 10% standard deviation in the delay t_m among individual spores).[30]

For subsequent evaluation of the secondary models, the maximum likelihood estimates for all the other parameters (except a) were obtained, and the information matrix for $\ln(\mu)$ and $\ln(t_m)$ estimated numerically for each temperature replicate at fixed values for C_0 for that temperature replicate, and for the experiment-wide C_m and the standard deviation of measurement errors.[31] This information matrix measured the variation in $\ln(\mu)$ and $\ln(t_m)$ to be expected based on the measurement errors only.

Mathematically, for a replicate (a single growth versus time curve at fixed temperature and identical initial conditions) with index i within an experiment (multiple growth curves, possibly including multiple replicates at each temperature), it is expected that

$$\ln\left(C_{ij}\right) = \ln\left(f\left(t_j, T_i, C_{0i}, \mu_i, t_{mi}, C_m, a\right)\right) + \varepsilon \qquad \textbf{(3.21)}$$

where C_{ij} is the CFU/g after time t_j in a replicate experiment at temperature T_i, f is the primary model, and ε is normally distributed error term with mean zero and standard deviation σ.

C_m, a, and σ are experiment-wide parameters, while C_{0i}, μ_i, and t_{mi} apply to this replicate (numbered i). The term σ represents the experimental error. The conditional loglikelihood for the expectation represented by Equation (3.21) (given C_{0i}, μ_i, and t_{mi}) is:[32]

$$J = \sum_i J_i = -\sum_{i,j}\left(\ln\sigma + \frac{\left(\ln\left(C_{ij}/f_{ij}\right)\right)}{2\sigma^2}\right)$$

where **(3.22)**

$$f_{ij} = f\left(t_j, T_i, C_{0i}, \mu_i, t_{mi}, C_m, a\right)$$

Now find maximum likelihood estimates for all the parameters, and compute the information matrix for each $\ln(\mu_i)$ and $\ln(t_{mi})$ at fixed values for the other parameters. Then for each replicate approximate the interesting part (*i.e.* just the part involving μ_i, and t_{mi}) of the conditional likelihood by a normal that looks like:

$$\exp\left(J_i\right) \sim \left|B_i\right|^{1/2} \exp\left(-x_i' B_i x_i\right)$$

where **(3.23)**

$$x_i = \left(\mu_i - \mu_i^*, t_{mi} - t_{mi}^*\right)'$$

[30] Further analysis testing the effect of varying values of a might be appropriate.

[31] This underestimates the uncertainties slightly through failure to take account of the co-variance of these other parameters. However, the effect appears to be small.

[32] It would be preferable to start with the experimental colony count data and explicitly convolve the poisson uncertainty associated with counts with an additional experimental uncertainty. The analysis given here corresponds to starting with estimates of CFU/g obtained from those colony count data.

and * denotes maximum likelihood estimate, ‘ denotes transpose, and B_i is the information matrix for $\ln(\mu_i)$ and $\ln(t_{mi})$.

The Ratkowsky equations for μ and $1/t_m$ (secondary models) were estimated by assuming that $\ln(\mu)$ and $\ln(t_m)$ have normally distributed variabilities about the Ratkowsky equations (in addition to their uncertainties of measurement). These variabilities are taken to represent the experiment-to-experiment variation in μ and $1/t_m$, and are subsequently used as surrogates for variations that are expected in different food media, between different strains, and under different conditions (except temperature). The variabilities are represented in the analysis by a variance-covariance matrix that allows evaluation of any correlation between the variation in μ and the variation in $1/t_m$. To estimate the parameters of the Ratkowsky equations, and the magnitude of the experiment-to-experiment variability variance-covariance matrix components, the total variation in $\ln(\mu)$ and $\ln(t_m)$ is estimated by a variance-covariance matrix equal to the sum of the experiment-to-experiment variability variance-covariance matrix, and the inverse of the information matrix representing experimental errors. All parameters of the Ratkowsky equations and the experiment-to-experiment variability variance-covariance matrix were then estimated by maximum likelihood. There are nine parameters involved for each experiment — $T_{\min}$, $T_{\max}$,[33] two parameters each for the Ratkowsky curves for each of μ and $1/t_m$, two variances and one covariance for the experiment-to-experiment variability.

Mathematically, it was assumed that

$$\begin{aligned} \ln(\mu_i) &= \ln\left(R\left(T_i, X_m, A_m, T_{\min}, T_{\max}\right)\right) + \eta \\ \ln\left(1/t_{mi}\right) &= \ln\left(R\left(T_i, X_t, A_t, T_{\min}, T_{\max}\right)\right) + \phi \end{aligned} \qquad \textbf{(3.24)}$$

where R is the secondary model (of Ratkowsky form) with parameters $T_{\min}$, $T_{\max}$,[34] X (location of maximum) and A (height at the maximum), with subscripts m and t distinguishing values for μ and t_m. The terms (η,ϕ) represent variability from replicate to replicate, and are assumed to be jointly normal with zero mean and variance-covariance

$$Q = \begin{pmatrix} s_m^2 & c_{mt} \\ c_{mt} & s_t^2 \end{pmatrix} \qquad \textbf{(3.25)}$$

Then the loglikelihood (not conditioned on μ_i, and t_{mi}) for replicate i can be approximated by the loglikelihood for a normal form with variance-covariance matrix $Q + B_i^{-1}$ (this comes from the relevant convolution integral over μ_i, and t_{mi}). Summing these over all replicates gives a loglikelihood for the whole experiment. The nine parameters $T_{\min}$, $T_{\max}$, X_m, A_m, X_t, A_t, s_m, c_{mt}, and s_t are then estimated maximizing that loglikelihood, and the uncertainties in the parameters (and the correlations between those uncertainties) by computing the inverse of their information matrix.

[33] Based on previously published analyses, $T_{\min}$ and $T_{\max}$ were assumed to be equal for the Ratkowsky equations for μ and t_m.

[34] It was assumed that the same maximum and minimum temperatures apply to the growth rate μ and the delay time t_m.

3.11.3. Results for growth rates of *C. perfringens* and *C. botulinum*

The experiments on cured chicken and cured beef (Juneja *et al.*, 2001; Juneja and Marks, 2002) give results for all 9 parameters that are statistically indistinguishable. The cooked ground beef data (Huang, 2003) provides maximum likelihood estimates that are distinct, but apparently largely because the analysis attempts to estimate 9 parameters from only 6 growth curves, each giving a μ and t_m estimate, but with no replicate information available at each temperature. The estimates of s_m, c_{mt}, and s_t obtained from these data appear to be anomalously low.[35] With the variance-covariance matrix forced to be identical to that obtained from the cured chicken and cured beef experiments, the maximum and minimum temperatures, T_{min} (12.5 °C), T_{max} (53.5 °C), and the shape parameters X_m and X_t for the Ratkowsky curves all agree with the cured beef and cured chicken ones, although there appears to be faster growth (by a factor of 1.9) and shorter times to start division (about 1.6-fold shorter). The difference in growth rate and time to start division are expected because of differences in growth media, so we adopted the analysis with variance-covariance matrix forced to be identical with the cured chicken and cured beef analyses. The broth data (Juneja *et al*., 1999) have statistically different T_{min} and T_{max} (13.6 to 54.1 °C). The Ratkowsky growth curve has the same shape as for beef and chicken, but a different amplitude; but the Ratkowsky curve for $1/t_m$ has a different shape. It seems plausible that the curve shape for growth is universal (for these *C. perfringens* strains), with growth rates dependent on experimental conditions; but the curve shape for $1/t_m$ (1/time-to-division) likely depends on activation methods (Figure 3-4 plots the Ratkowsky growth-rate versus temperature curves with parameter values estimated from the data).

It was judged that the most representative estimates for parameters for use in this risk assessment are those corresponding to the cooked cured beef and cooked cured chicken experiments, modified as described below. The parameter estimates for cooked ground beef are similar, but with higher growth rate and shorter delay period, as would be expected for conditions that are probably close to ideal for the *C. perfringens* strains used. The parameter values estimated for cooked cured beef and cooked cured chicken are given in Table 3.23:[36]

Table 3.23 Maximum likelihood estimates for growth parameters for *C. perfringens* in cooked cured beef and cooked cured chicken.

T_{min} (°C)	12.5
T_{max} (°C)	53.5
A_m (per hour)	2.084
X_m	0.250
A_t (per hour)	0.455
X_t	0.193

[35] This may partly be because at least some of the data are averages of up to three experiments, but such averaging cannot be the whole explanation.

[36] Jointly estimated with those for cooked ground beef, with only the amplitudes of the Ratkowsky curves allowed to differ for the cooked ground beef.

s_m	0.347
c_m	0.046
s_t	0.362

Note: parameters are defined in Sections 3.11.1and 3.11.2

The uncertainties in these parameters are given in the standard-deviation/correlation matrix shown in Table 3.24.

Table 3.24 Standard deviations (diagonal) and correlation coefficients (off-diagonal) for the parameter estimates of Table 3.23

$T_{\min}$ (°C)	0.211	0	0	0	0	0	0	0	0
$T_{\max}$ (°C)	-0.050	0.912	0	0	0	0	0	0	0
A_m (per hour)	0.217	0.116	0.128	0	0	0	0	0	0
X_m	0.150	0.226	-0.157	0.005	0	0	0	0	0
A_t (per hour)	-0.073	-0.280	-0.341	0.004	0.046	0	0	0	0
X_t	0.293	0.601	0.164	0.091	-0.692	0.026	0	0	0
s_m	0.027	0.015	0.058	0.047	-0.006	0.020	0.040	0	0
c_m	-0.092	-0.044	0.057	-0.023	0.165	-0.127	0.502	0.026	0
s_t	-0.031	-0.017	-0.020	-0.009	0.228	-0.082	0.154	0.552	0.050

Note: parameters are defined in Sections 3.11.1and 3.11.2

The *C. botulinum* data (Juneja and Marks, 1999) give ($T_{\min}$, $T_{\max}$) as 8.2 to 50.03 °C, where additional constraints based on no observed growth for 11 weeks at 11 °C and 50 °C have been applied (in the likelihood estimation) at these temperatures, by specifying $t_m > 504$ hours in both cases (using the Ratkowsky curve prediction). It is likely that the Ratkowsky curve shape is not ideal at either end of the range of temperatures, so this strong constraint at the top end may distort the estimated curve away from the data. [37]

[37] L. Huang has pointed out (personal communication, 2004) that the estimated upper temperature limit for *C. perfringens* may be too high, based on his unpublished laboratory observations. The maximum temperature for which data are reported in the literature is 50 °C (at which temperature growth still occurred), and no limits on growth rate was identified for higher temperatures. Thus for *C. perfringens* the estimated T_{max} is an extrapolation based on the Ratkowsky curve, which may have the wrong shape near T_{max}. For *C. botulinum*, the estimation procedure incorporated a published stringent bound on growth rate at 50 °C. The qualitative feature of a small range of temperatures around and above 50 °C where the growth rate of *C. perfringens* substantially exceeds that of *C. botulinum*, or where *C. perfringens* can grow but *C. botulinum* cannot, is solidly based in observations.

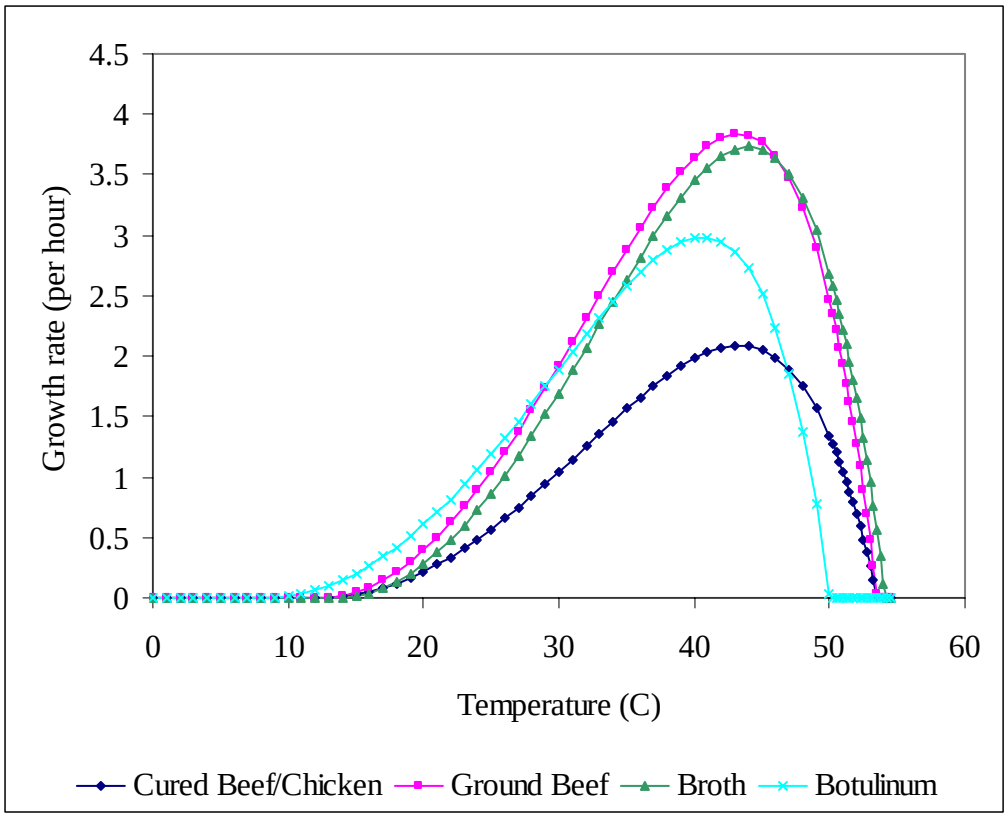

Figure 3-4 Average growth rates of *C. perfringens* in the three media indicated, and of *C. botulinum* in a laboratory medium, and how these rates are estimated to vary with temperature.

3.11.4. Comparison with published growth rates.

The results of Section 3.11.3 apply strictly to just the experiments analyzed. Those experiments were performed on a mixture of three strains of *C. perfringens*, under tightly controlled conditions. The variations between them may therefore underestimate the variations to be expected between growth conditions and strains in RTE and partially cooked foods. In an attempt to evaluate any bias in the results, and to identify any major additional variability, a literature review of growth rates was conducted, to construct a compilation of 174 reported measurements of generation times for *C. perfringens* within meat foods. This compilation includes almost all measurements that could be identified.[38] The measurements generally were for cooked meat, but include some measurements on raw meat. However, no results were included that resulted from experiments in liquid media or only on the surface of meat. The strains used were identified as:

[38] One reference, Naik & Duncan (1977), was obtained too late for inclusion. Smith (1963) includes a graph showing generation times for 5 unidentified strains at 5 °C temperature intervals from 20 °C to 50 °C that was recognized too late for inclusion.

1362
5 strain composite (NCTC 8679, 8238, 8239, R42, PS44)
8 strain composite (NCTC 8238, 10240, 8797, 8798, 8239; ATCC 3624; S-40, S-45)
8-strain composite (NCTC 8238, 10240, 8798, 8239, 9851; ATCC 3624; S-40, S-45)
ATCC 3624
F2985/50
FD-1
FD-1041
NCTC 8238
NCTC 8239
NCTC 8797
NCTC 8798
S40
S45

The measurements were at temperatures varying from 12 °C to 51 °C . Some of the estimates obtained have considerable uncertainty, since they were obtained from just two points, and/or were obtained by digitizing graphs in the papers.

To compare with the results of Section 3.11.3, the ratio of observed to predicted generation time was constructed, where the "predicted" value is that obtained using the parameters given in Table 3.1. Figure 3-5 shows the distribution of the logarithm of observed to predicted generation times on a normal scale. There are exactly 3 outliers where the model predicts growth rates much lower than observed (generation times much longer). All three are at low temperatures.

- 12 °C, Solberg and Elkind (1970). The observed generation time is 580 minutes, estimated from Figure 5 of the paper, with a model estimate of zero growth (this is shown on Figure 3-5 with a generation time arbitrarily set to 50,000 minutes). This is the only available measurement at such low temperatures (although there are several reports of no growth at 10 °C).

- 15 °C, Juneja *et al.* (1994b). The observed generation time is 43.2 minutes (strain NCTC 8238), with a model estimate of 1660 minutes.

- 15 °C, Juneja *et al.* (1994b). The observed generation time is 43.2 minutes (strain NCTC 8239), with a model estimate of 1660 minutes.

Four other measurements at 15 °C were located in the literature, three of them by Juneja *et al.* (1994b) with the same strains, one by Solberg and Elkind (1970), where the model also underestimates growth rate (1660 minutes generation time) but not so drastically. The next higher temperature measurement located in the literature is 20 °C.

There are 6 cases where the model predicts growth rates substantially (>1.6-fold, but see below about bias) larger than observed. They are not listed here because such overestimates are conservative for the risk assessment (leading to overestimates of risk).

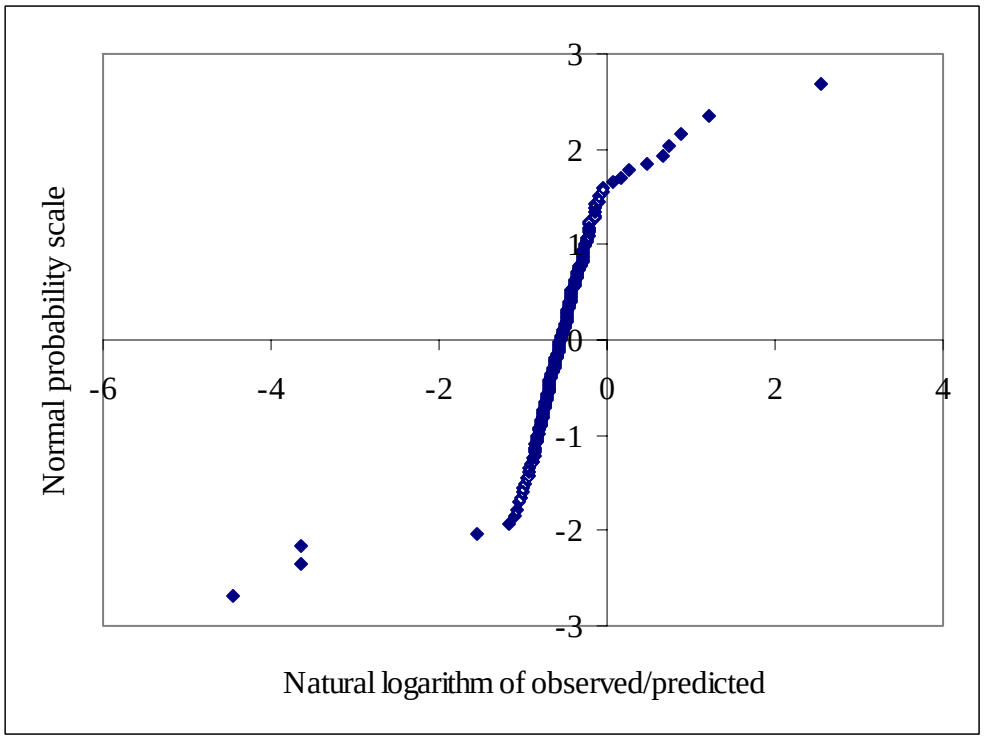

Figure 3-5 Empirical distribution of natural logarithm of observed/predicted ratio of generation times for *C. perfringens* (the most extreme outlier on the left is placed arbitrarily; predicted growth rate is zero, but growth was observed).

The remaining 165 observed/predicted ratios form a lognormal distribution (p=0.55, Shapiro-Wilk statistic; and they look almost exactly straight on a normal probability plot). The median observed/expected generation time is 0.575, so the model generally underestimates published growth rates by about a factor of 1.739. The standard deviation of ln(observed/predicted) is 0.27 (1.3-fold), which is smaller than the similar standard deviation (s_m = 0.35 ± 0.04, 1.4-fold) estimated for the between-experiment variation in the analysis of experiments in Section 3.11.3 (see Table 3.23 and Table 3.24).

The model appears to estimate generation time (growth rate) well, with the following reservations and modifications:

1. The values for growth rates obtained in Section 3.11.3 may be biased to underestimate growth rates. It was considered that the compendium of all published data is more likely to be representative of the distribution of strains and conditions to be expected in meat and spices entering the RTE and partially cooked food chain than the selected experiments analyzed in Section 3.11.3 (since they were selected by their availability and for the quality of data available for analysis, not their representativeness). All modeled growth rates are therefore increased by a factor of 1.739 to agree with the median of published data[39] (omitting outliers[40]). This should be

[39] This adjustment was added to the model as a lognormal distribution with median 1.739 and an standard error (estimated from the data) of a factor of 1.02.

conservative, although it may not be correct. It is possible that many reported experiments were performed with strains selected to be the fastest growing available, so published generation times may systematically be lower than would be expected for representative selections of strains and conditions.

2. The between-experiment variability in logarithm of growth rate (s_m, Section 3.11.3) estimated in the model fit is large enough to represent the between-situation (between conditions, between strains) variation seen in the published estimates of growth rates. No adjustment to this variability was made.

3. The model may underestimate growth rates at low temperatures, below about 20 °C; this underestimation may come about because of the imposed shape of the Ratkowsky curve — the same underestimation is apparent in the analysis of the experiments of Section 3.11.3. The model predicts no growth below 12.5 °C, but growth has been observed at 12 °C (Solberg and Elkind, 1970). There are very few published data allowing estimates of growth rates below 20 °C.

No similar comparison could be made of estimates of the delay time before exponential growth occurs after heat shock to spores, since there are few such estimates available in the literature. There is some evidence (Juneja and Marks, 2002) that growth rate and delay time are inversely proportional within individual experiments, although the between-experiment variations in growth rates and delay times are practically uncorrelated (see Table 3.24). In view of this evidence, the delay time estimated by the model is similarly decreased by the same factor (median 1.739) as the growth rate is increased. This has very little effect on the modeling performed in this risk assessment, except for the estimates for hot-holding, where it may result in a conservative bias (towards overestimates of illnesses).

The between-experiment variation of delay time is assumed to be adequately represented by the estimates of Table 3.24. A complete accounting for variability would explicitly take account of the likely probabilistic nature of initial cell divisions. However, the measured between-experiment variation incorporates such stochastic variation corresponding to the spore densities used in the experiments. Such variability is probably be spore-density dependent (the relative variation increasing at lower spore densities), and most experiments have been with spore densities of around 100 CFU/g. It appears that the major contribution to risk estimates comes from initial spore densities that are lower than 100 CFU/g, so variability of delay times may be underestimated by the between-experiment variation. The extrapolation between spore densities used in growth experiments and those occurring in naturally contaminated servings may thus result in an underestimate in variability in the growth achieved in hot-holding situations[41]. Moreover, the modeling does not incorporate any spore-density-dependent variation of the variability, as would be expected if the initial cell divisions are probabilistic in nature.

[40] Outliers were identified initially by eye from Figure 3-5, then confirmed by noting that inclusion of any of them reduced the Shapiro-Wilk statistic (testing for departure from a lognormal distribution) to less than 0.10.

[41] The delay time does not affect any other part of the model for RTE and partially cooked foods, since it is not explicitly used elsewhere.

3.11.5. Modifications of growth rate by environmental factors

It is expected that the growth rate of *C. perfringens* is influenced by factors other than the temperature. As an example, as the salt and nitrite content of RTE foods increases, it is expected that *C. perfringens* growth is slowed. Similarly, a more acidic environment (low pH) is expected to slow *C. perfringens* growth. Low water activity is expected to slow or halt *C. perfringens* growth. Expectations aside, the challenge for this analysis is to quantify the influences of these physical/chemical factors on *C. perfringens* growth rates.

3.11.5.1. Presence of oxygen.

There is substantial evidence that the presence of oxygen influences the growth of *C. perfringens* in foods (Juneja *et al*., 1994a; Hintlian and Hotchkiss, 1987). Exposure to atmospheric levels of oxygen strongly inhibits the growth of this anaerobic bacterium. However, the manufacturing heat treatment drives off much of the oxygen and thereby provides an acceptable atmosphere for *C. perfringens* to grow. Many RTE foods are cooked in, or rapidly placed in, casings or packagings that help maintain an anaerobic environment. The presence of oxygen was therefore not incorporated into the growth model.

3.11.5.2. Salt and Nitrite effect on growth rate

The presence of nitrites and salt in an RTE food commodity is considered inhibitory of *C. perfringens* growth at levels of 3% salt or greater (see Appendix A). For foods containing nitrite but salt concentrations less than 3%, slower *C. perfringens* growth may occur. For instance, in the range of 1–3% salt, *C. perfringens* growth was slowed in cured and uncured turkey emulsion (Kalinowski *et al.*, 2003),[42] and inhibition by salt (0–2%) of *C. perfringens* growth in a broth mixture including sodium pyrophosphate was also apparent (Juneja *et al.*, 1996b).

To estimate the effect of low salt concentrations in food on the growth of *C. perfringens* the reported data of Kalinowski *et al.* (2003) and Juneja *et al.* (1996b) were examined. The primary growth model was fitted to the data of Kalinowski *et al.* (2003, tables 4 and 5) in cured (156 μg/ml sodium nitrite) and uncured turkey with 1% salt, and a relative growth rate at 2% and 3% salt and 43.3 °C estimated based on the single log(CFU/g) data points published for these salt concentrations and temperature (no growth was observed in the cured turkey at 3% salt). These point estimates of relative growth rate were: 2% — 0.69; 3% —0.17. Juneja *et al*. (1996b) performed 90 experiments with 45 combinations of conditions according to a partial factorial design for growth of *C. perfringens* in a broth with 0–3% salt, pH 5.5–7, sodium pyrophosphate 0–0.3%, at five temperatures in the range 12–42 °C. They fitted Gompertz models and estimated kinetic parameters from the Gompertz parameter estimates. The published data on exponential growth rate (EGR) were compared with the estimated growth rates at corresponding temperatures from the primary model (Section 3.11.3), and the logarithm of the ratio of these two fitted with a model that included linear and quadratic terms in salt concentration, pH, and pyrophosphate concentration, products in pairs of temperature, salt concentration, pH, and pyrophosphate concentration, and a normal error term (corresponding to the quadratic models of Juneja *et al.*, 1996b, but with all temperature-only terms omitted, since the temperature effect is modeled by the primary growth model). All terms except the linear and quadratic pyrophosphate

[42]Although *C. perfringens* growth may be inhibited by concentrations of salt <2%, no studies were identified to confirm this.

term, the temperature-pyrophosphate interaction term, and the quadratic salt term, were non-significant and dropped. The effect of salt could thus be estimated as

$$\ln(R) = k - \lambda S^2 \qquad \textbf{(3.26)}$$

where the terms are

- R ratio of EGR to growth rate μ predicted by the primary model of Section 3.11.3,
- S salt percentage in the broth,
- k a constant accounting for different units of measurement and different conditions for the experiment, and
- λ a coefficient measuring the effect of salt.

The estimated value of λ is 0.179 ± 0.064 (uncertainty standard error; the profile likelihood is very well modeled by a normal distribution) per (salt %)^2, and this value gives estimates for the ratios of the effects at 2% and 3% relative to 1% of 0.58 and 0.24 respectively, consistent (taking account of the uncertainty) with the observations of Kalinowski *et al*. (2003), suggesting that the effect is relatively independent of the growth substrate (ground turkey versus a laboratory broth medium). This estimate for λ is used in the risk assessment, and applied to all foods based on their salt content.

Low concentrations of nitrite appear to affect growth rates independently of salt content, although few data were located to measure the effect quantitatively. Kalinowski *et al*. (2003, tables 4 and 5) report growth curves from spores in cured (156 μg/ml sodium nitrite) and uncured turkey emulsion at 26.7, 32.2, 37.8, 43.3, and 48.9 °C, both at 1% salt content. Growth at the two lower temperatures was substantially suppressed; although some initial growth occurred, the concentration never increased 10-fold, and the measurements are consistent with zero growth. At temperatures closer to the optimum growth temperature, growth rates were reduced by 30–50%

To take some account of the effect of nitrite, the ratio of growth rates for the three higher temperatures was evaluated to be 0.582 ± 0.042 (uncertainty standard error; the profile likelihood is very well modeled by a normal distribution). This factor is applied to the estimated growth rates of *C. perfringens* in all Category 1 foods (nitrite-containing) at all salt concentrations and at all temperatures, since it is not known whether the apparent suppression of growth by larger factors occurring at 1% salt and larger temperature deviations from optimum growth conditions would also occur at other salt concentrations.

3.11.5.3. The effect of salt and nitrite on the length of delay time

Few data were identified to estimate the delay time before growth in the presence of combined salt and nitrite in food. In their study, Juneja *et al.* (1996b, see Section 3.11.5.2) evaluated the effect of salt, temperature, sodium pyrophosphate, and pH in a laboratory broth medium. Salt appeared to be significant in various interaction terms in a model for lag phase duration (estimated from fitting Gompertz curves to experimental growth data). However, the delay time in broth is significantly longer than that in food-like meat media, so the application of these results to RTE and partially cooked foods is questionable. This risk assessment assumes that salt has no effect on the delay times.

Riha and Solberg (1975) estimated the lag phase of heat-resistant strain *C. perfringens* strain NCTC 8797 in laboratory media that contained nitrite only (Table 3.25).

Table 3.25 Mean lag phase and generation time of *C. perfringens* NCTC 8797 at 43 °C. (Riha and Solberg, 1975).

Nitrite concentration (ppm)	No. of lag experiments	Lag phase duration (hrs)	No. of generation experiments	Generation time (min)
0	10	7.8	9	23.9
100	8	10.2	7	25.4
150	4	9.5	8	23.2
175	4	9.8	4	30.3
200	4	-[a]	1	16.2

a. No growth observed for 60 hours.

These data suggest little effect of nitrite alone on lag phase duration. Kalinowski *et al.* (2003) report growth curves from spores in cured (156 μg/ml sodium nitrite) and uncured turkey emulsion at 26.7, 32.2, 37.8, 43.3, and 48.9 °C, both at 1% salt content. When these data are fitted using the primary growth model of Section 3.11.1, there is no significant difference between the delay times in cured and uncured turkey.

Labbe and Duncan, (1970) showed that the length of the lag phase of the same *C. perfringens* strain was increased in the presence of 200 ppm nitrite (Table 20). Riha and Solberg (1975) performed experiments in filter sterilized media and suggested that the long lag times were attributable to inhibition by oxygen, as lag times in autoclaved media were about half those in filter-sterilized media. Unfortunately, autoclaving nitrite has been shown to result in a product that is more inhibitory to *C. perfringens* than non-autoclaved nitrite (Perigo and Roberts, 1968; Riha and Solberg, 1973). Therefore, the data on autoclaved nitrite could not be reliably incorporated into the growth model.

Table 3.26 Lag phase of *C. perfringens* NCTC 8798 at 45°C (Labbe and Duncan, 1970).

Nitrite concentration (ppm)	Lag phase duration (mins)
0	~35
100	~45
200	>105[a]
[a] Final sample.	

The available data on nitrite are thus equivocal; however, the only available data indicating an increase in delay time are in laboratory media, and have not been analyzed taking account of all

the uncertainties in measurements. For this risk assessment, no change in delay time will be modeled for nitrite.

3.11.5.4. The effect of pH

There is some evidence to suggest that *pH* affects germination rates of *C. perfringens* spores. Experiments using heat-resistant spores of *C. perfringens* showed that as the pH of the solution increased, the optimal temperature for germination decreased (Craven, 1988). For instance, optimal germination was observed for spores at pH 5.6 and 75 °C for 20 minutes. However, at pH 5.6, germination fell by 2.3 fold at 65 °C. At pH 6.6, a similar fraction of germinated spores was observed after both 65 and 75 °C for 20 minutes. However, in these studies Craven (1988) quantified change in germination by measuring reduction of optical density values rather than by enumeration; and the relation of this measurement to the delay time modeled here is not known. Consequently, any separate effect of *pH* on germination of *C. perfringens* spores could not be reliably modeled in this risk assessment

Juneja *et al.* (1996b) showed significant effects of *pH* on lag phase duration and generation time (both estimated from Gompertz fits to experimental growth curves) for *C. perfringens* growing in a laboratory broth medium containing salt and sodium pyrophosphate. An analysis of their published estimates of exponential growth rates (see Section 3.11.5.2) showed no significant effect of *pH*. No further information was located that would allow estimates of the effect of *pH*. Since there appears to be no effect of *pH* (for a reasonable range of values) on exponential growth rates (the closest match to the growth rate parameter used in the primary model used here), this risk assessment does not model any effect. Delay times may be affected in laboratory broth media, but the relevance of that finding to food-like meat media is not clear since delay times differ between these two media types. This risk assessment does not model any effect of *pH* on delay times.

3.11.5.5. Water activity

Water activity refers to the water available for biological processes. Kang *et al*. (1969) grew heat-activated *C. perfringens* spores in laboratory media with varying water activity. The water activity levels were controlled by the addition of three solutes (glycerol, sucrose, and sodium chloride) in separate experiments. In a test that could not distinguish germination alone from germination plus growth, spores germinated and grew approximately equally over 24 to 48 hours in glycerol adjusted water activities from 0.95 to 0.995, with some germination at water activities down to 0.94. In sucrose or sodium chloride adjusted media, germination and growth was demonstrated over the somewhat narrower range from a water activity of 0.96 upwards. Studies with *C. botulinum* indicate that spores are able to germinate at water activity levels below those that permitted growth of vegetative *C. botulinum* cells (Baird-Parker and Freame, 1967; Williams and Purnell, 1953). It is therefore reasonable to suppose *C. perfringens* spores capable of germinating below the level of water activity allowable for vegetative cell growth.

In other experiments that followed the vegetative cell growth curves from *C. perfringens* heat-activated spores, Kang *et al.* (1969) demonstrated growth in water activities of 0.97 and above, and consistently declining concentrations at 0.93 or lower water activities. A water activity of 0.95 gave growth in glycerol adjusted media, but declining concentrations in sucrose and sodium chloride adjusted media. The growth curves indicate longer delay times in sucrose and sodium

chloride adjusted media at the lower water activities, possibly combined with slightly reduced growth rates. In glycerol, there were slightly reduced growth rates or slightly longer delay times or both at lower water activities, but the experimental measurements are inadequate to distinguish these.

Generally, these data suggest that growth rate is not substantially affected at water activities at or above 0.97, but that die-off of organisms begins to occur at or below a water activity of 0.93, with media-dependent results at 0.95 water activity. It is unclear whether low water activities levels kill heat activated spores, or return them to an inactive state. For this risk assessment, it is assumed that water activity levels at or below 0.93 suppress growth completely, but no lethality from such low water activities occurs. Above 0.93, it is assumed that growth rates and delay times are unaffected, despite the observation of somewhat longer delay times and/or lower growth rates. The assumption of no effect is justified because the observations could be explained solely by slightly longer delay times, yet such delay times are media-dependent and appear smaller in food-like meat media compared with laboratory liquid media.
Water activity values for foods compiled from the literature (Table 3.27) are all above 0.95, so in this risk assessment no adjustment for water activity is applied.

Table 3.27 Water activity values of meat items (Chirife and Ferro Fontan, 1982; Alzamora and Chirife, 1983; Taormina *et al*., 2003; Fett, 1973).

Sample	Chirife and Ferro Fontan, 1982	Alzamora and Chirife, 1983	Taormina *et al*., 2003	Fett, 1973
Beef	0.98-0.99			
Beef Corned		0.972, 0.979		
Roast beef		>0.982		
Bologna, raw			0.965, 0.965	
Bologna, cooked			0.966, 0.952	
Pork	0.99			
Pork sausage				
Measurement method 1				0.99, 0.97
Measurement method 2				0.973, 0.973
Ham, cooked		0.971		
Ham, deviled		0.971, 0.970, 0.975, 0.977		
Ham, chunked raw			0.973, 0.977	
Ham, chunked cooked			0.964, 0.967	

Ham, whole muscle raw			0.979, 0.985	
Ham, whole muscle cooked			0.972, 0.978	
Chicken, boned		0.982		

3.11.5.6. The maximum vegetative cell density

In evaluation of the experiments of growth rates of vegetative cells derived from heat-shocked spores (Sections 3.11.2 and 3.11.3) the maximum cell density was assumed to be identical for all growth conditions within each set of experiments described by the various authors. The estimated values obtained for the maximum vegetative cell densities were 9.9 $\log_{10}$ (experiments of Juneja *et al.*, 1999) using a broth medium; 7.6 $\log_{10}$ (experiments of Juneja *et al.*, 2001) in cooked cured beef; 8.07 $\log_{10}$ (experiments of Juneja and Marks, 2002) in cooked cured chicken; and 8.03 $\log_{10}$ (experiments of Huang, 2003) in cooked ground beef. No formal analysis was performed of the variability between these values, nor was any attempt made to account for potential differences between the experiments at different temperatures reported in each study.[43] It is expected that different foods, with different meat fractions, could have substantially different maximum possible *C. perfringens* vegetative cell densities, but little information was identified in the literature that would allow testing of such a hypothesis. To encompass the differences observed in the laboratory experiments performed on meat media (the high value measured in broth was discounted), it was assumed that the maximum cell density in all foods is 8 $\log_{10}$, with a variability of 0.5 on the $\log_{10}$ scale. The effect of this assumption is tested in the sensitivity analysis.

3.12. Growth during chilling, stabilization and secondary cooking steps — the factor G_c

The amount of growth allowed during chilling, stabilization, and secondary cooking steps is the proposed control variable for regulations, and so must be modeled as an input to the risk assessment in some fashion. A fully realistic evaluation of the effect of different regulations would require knowledge of a mapping between the regulatory level of growth allowed, and the distribution of the amount of growth achieved in practice in all RTE and partially cooked foods. We do not have that mapping, nor do we have the information needed to model it — we do not have, for example, the extensive information on the cooling curves that would be used in the industry under various regulatory regimes (indeed, we are unable to say what is the current distribution of growths achieved under the current regulatory regime).

Given these circumstances, we opt for an approach that can provide some information, although not necessarily the exact information desired. In the implementation of the model, the option is provided of specifying any variability distribution for growth. Thus it is possible to specify a single value for the growth experienced by all RTE and partially cooked foods (using a point distribution), or a distribution of values corresponding to the possible range of values that would

[43] The maximum cell density appeared to be homogeneous between temperatures, except for the 50 °C experiment in Huang (2003), where the maximum cell density tested as significantly lower than at lower temperatures; however, this difference was ignored in the analysis.

be achieved in practice for a given regulation. The results we present correspond to using fixed values of growth (G_c chosen to be a fixed value, typically with $\log_{10}G_c$ equal to 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, or 3.5), with the full realization that they do not correspond exactly to any regulatory regime.

3.13. Storage and transport phases of the distribution system for RTE foods

Once an RTE product is manufactured and has been stabilized, it is distributed to the final consumer for preparation and consumption. Nevertheless, distributing RTE products from a relatively small number of producers to a very large number of consumers results in possibly long periods of storage. Typically, the product must move from the manufacturing plant to a retail store; then move to a consumer's refrigerator. Some degree of spontaneous germination of spores remaining in the products is expected and the data used to assess this are described in this section. Additionally, during the period between manufacture and preparation, the product may be stored at some temperature(s) that could allow growth, retard growth or cause cell death. These temperatures and the associated times are also discussed in this section.

3.13.1. Spontaneous germination of spores during storage and transport — the fraction g_s

Spores that remain in RTE or partially cooked products after chilling may spontaneously germinate during storage of the products. For simplicity, conservatism, and because this is expected to be a minor contributor to risks, it is assumed in the model that all spontaneous germination takes place at the beginning of any such storage.

Section 3.9.5 summarized the available evidence on germination of spores without heat activation. As noted there, even under frozen storage a visible fraction of spores germinated after 1 or 2 months (Ahmed and Walker, 1971). Most reported result were, however, under conditions that were presumably more favorable to germination than typical storage conditions for RTE and partially cooked foods.

To encompass the measurements described in Section 3.9.5, but taking account of the harsher expected conditions, the fraction g_s of Type A, CPE positive strains germinating in storage is modeled in the same way as for the fraction germinating under favorable conditions using a triangular distribution ranging from 0 to 5%, with mode 2.5%. A sensitivity analysis is performed on these parameters and distribution shape to determine the effect of this set of assumptions. The fraction germinating was also assumed independent of the temperature, duration, or any other conditions of storage.

3.13.2. Survival or growth of *C. perfringens* during storage and transport — the factor G_s

C. perfringens is inhibited from growing below about 10 °C, but lower temperatures can be lethal. Because standard RTE food chilling practices typically attain temperatures below 5 °C, and storage of RTE and partially cooked products is usually at temperatures below 10 °C (see Section 3.13.3), the lethal effect of low temperatures is included in this model for temperatures below T_{min}. Above T_{min}, the expected growth rates for such higher temperatures are applied (Section 3.13.2.4). The factor G_s of Equation (3.3) is obtained as the product of two factors, one for each period of storage (Section 3.2). The factor for each period is obtained by applying the respective growth or death rate for the corresponding temperature and time (Section 3.13.3).

Available evidence indicates that *C. perfringens* exposed to low temperatures cannot multiply; but rather the cold may kill *C. perfringens* vegetative cells. The exact mechanisms responsible for cold shock lethality are not clear, although freezing of bacterial cell membrane lipids is likely critical (Leder, 1972). Low temperatures could therefore reduce the concentration of *C. perfringens* vegetative cells within a RTE or partially cooked commodity. To evaluate the effect of cold on *C. perfringens* survivability in foods, the following factors were evaluated: (1) cooling during the bacterial growth phase, (2) duration and temperature, and (3) food composition.

The effect of cold shock following bacterial growth
Bacterial growth may be inhibited due to injury and/or death of bacterial cells following chilling in a medium. Vegetative *C. perfringens* cells that are growing exponentially are more susceptible to killing by cold than those that are not in this growth stage. Traci and Duncan (1974) reported that 96% of exponentially growing *C. perfringens* cells were killed upon cold shock at 4 °C. Moreover, 95% of the remaining cells were killed following 90 minutes exposure at 4 °C. In contrast, a greater number of cells in stationary phase remained viable following cold shock.

C. perfringens are likely to experience a several hour cooling process plus a stabilization process at the manufacturing plant (although more rapid cooling processes are in use in some cases). Bacteria exposed to these conditions are not likely to be in exponential phase and may be less susceptible to cold lethality than exponential phase cells.

Duration and temperature of storage
Both the duration and the temperature to which *C. perfringens* are exposed affect the bacteria's survivability. There are indications that freezing temperatures can be less detrimental to *C. perfringens* vegetative cells than refrigeration temperatures (Barnes *et al.*, 1963; Strong *et al*., 1966). It also appears that most killing of *C. perfringens* by cold occurs rapidly, affecting the most susceptible cells and leaving more cold-resistant cells. Blast freezing is typically used to freeze foods such as those listed in Category 3. Data from Barnes *et al.* (1963) suggest blast freezing may result in as much as a 1 $\log_{10}$ reduction in the number of *C. perfringens* vegetative cells. However, the methodology used in this experiment was not reported in sufficient detail to discern whether it was similar to the blast freezing protocols used by industry. Consequently, for this risk assessment, the affect of blast freezing of *C. perfringens* vegetative cells in foods was not modeled.

Food composition
The composition of a product may affect cold lethality of *C. perfringens* vegetative cells. The data of Kalinowski *et al*. (2003) suggests that the presence of nitrite in ground turkey might increase the effect of cold lethality. However, other factors may also account for the differences, as discussed below, and for this risk assessment, lethality due to refrigeration is modeled similalry for all food compositions.

3.13.2.1. Selection of studies on the lethal effect of low temperatures

A number of studies were analyzed to provide evidence of the magnitude of the lethal effect cold temperatures have on *C. perfringens* vegetative cells in foods (Table 3.28). Only studies that examined survival in food matrices were used for evaluation purposes. In all the studies examined, concentrations of *C. perfringens* decreased during storage in a way that was consistent

with a regular exponential decrease with time (although other possibilities cannot be ruled out). In cases where cells were subjected to very rapid cooling just before the storage period began, there appeared to be an initial additional killing of cells — the zero-storage-time measured concentration in such cases was ignored in the analyses.

Taormina *et al*. (2003) measured concentrations of *C. perfringens* (an initially equal mixture of spores of 5 strains; ATCC 3264 [CPE negative], ATCC 12916 [CPE positive], FD1041 [CPE positive], and two strains isolated from meat product blends with unknown CPE status) through simulated commercial cooking, chilling, and storage in bologna (cured), ground cured chunked ham with emulsion, and ground cured whole-muscle ham. Storage was at 4.4 °C for 14 days, with concentration measurements immediately after completion of chilling (temperature 7.2 °C) and at 2 days, 7 days, and 14 days. There was no initial killing effect from rapid cooling in this case.

Barnes *et al*. (1963) inoculated about 10^5 vegetative cells of *C. perfringens* strain F2985/50 into raw beef blocks pre-sterilized by radiation and kept at 1 °C in impermeable bags. The vegetative cells were prepared by dilution in RCM broth from a culture grown in Robertson's cooked meat for 24 or 48 hours, so were probably in stationary phase. Storage was at –5 °C or –20 °C for 26 weeks, with measurements immediately after blast freezing and at 3, 5, 8, 12, and 26 weeks. There was an initial killing effect from the blast freezing, but the analysis here omits pre-blast-freezing measurements.

Kalinowski *et al*. (2003) inoculated approximately 100 spores/g of a mixed spore culture (strains NCTC 8239, NCTC 8798, NCTC 8449, and ATCC 13124 into raw cured or uncured turkey breast emulsion in vacuum sealed pouches. The pouches were cooked to 73.9 °C in flowing steam, cooled and held at 42 °C for 2 hours, then held at 0.6, 4.4, or 10 for 7 days, with sampling daily for 4 days and on the final day. The effect of cold shock was not measured. As discussed below, the vegetative cells (germinated from the spores) were probably in exponential phase.

Juneja *et al*. (1994a) inoculated approximately 1000 CFU/g of centrifuged and re-suspended stationary phase cell culture of strain NCTC 8239 into cooked ground beef in filter stomacher bags. Half the bags were vacuum packed in plastic barrier bags to maintain anaerobic conditions. Storage was at temperatures of 4, 8, and 12 °C, with measurements at days 0, 4, 8, 16, 24, 32, and 40. The effect of cold shock was not measured. Other temperature and time conditions resulting in growth are not analyzed here. There was no apparent distinction between aerobic and anaerobic conditions, and both were included in the analysis

Juneja *et al*. (1994b) inoculated approximately 1000 CFU/g of centrifuged and re-suspended stationary phase cell culture of strains NCTC 8238 and NCTC 8239 into cooked ground turkey in filter stomacher bags. Half the bags were vacuum packed in plastic barrier bags to maintain anaerobic conditions. Storage was at a temperature of 4 °C, with measurements at days 0, 6, 12, 18, 24, and 30. Results were reported for anaerobic conditions for NCTC 8238, and for both strains for aerobic conditions. Both aerobic and anaerobic results are included, since there was no apparent distinction. The effect of cold shock was not measured. Other temperature and time conditions resulting in growth are not analyzed here.

Strong and Canada (1964), in separate experiments, cultured five strains (8799F 1546/52, 214D, 65,108, and 142A) of *C. perfringens* in chicken gravy for 6 hours at 37 °C, sealed 1 ml. samples in glass tubing, and froze those samples at –17.7 °C. Samples were enumerated at 1, 2, 3, 10, 20, 30, 60, 90, 120, 150, and 180 days, but only enumerations at 1,10, 30, 90, and 180 days are reported and analyzed here.

Three other studies reported in Table 3.28 were omitted from the analysis, either because too few data were reported above detection limits or because only non-food media were used.

Table 3.28 Measurements on survival of *C. perfringens* vegetative cells under freezing and refrigeration conditions.

Reference	strain	Storage time (days)	Media
Taormina *et al*., 2003	CPE+:FD1041, ATCC12916; CPE-:ATCC 3624; and two CPE unknown strains	0–14	Ground bologna, chunked ham, and whole-muscle ham, all w/ nitrite
Barnes *et al.*, 1963	Heat-resistant, F2985/50[b,c]	0–182	Raw beef blocks
Kalinowski *et al*., 2003	Heat-resistant, NCTC 8239, 8798, 8449 and ATCC 13124[f]	0–7	Cured and uncured turkey
Juneja *et al.*, 1994a	Heat-resistant, NCTC 8239	0–40	Cooked ground beef
Juneja *et al.*, 1994b	Heat-resistant NCTC 8238 and 8239	0–32	Cooked ground beef
Stiles and Ng, 1979[a]	Heat-resistant, NCTC 8339-H	0–30	Sliced ham
Strong and Canada, 1964	Type A, 8799F 1546/52[b,d], 214D[b,d], 65[d],108[d], 142A[d]	0– 180	Chicken gravy
Raj and Liston, 1961[a]	*C. perfringens*	0– 393	Lab media and fish homogenate
Solberg and Elkind,1970[e]	Heat-resistant, S-80	3–83	Distilled water
Traci and Duncan, 1974[e]	Heat-resistant, NCTC 8798	0–0.04	Lab media

a. Too few data above detection limits for analysis.
b. *C. perfringens* strains isolated from food implicated in food poisoning.
c. *C. perfringens* grown for 24-48 hrs in Robertson's cooked meat before dilution in RCM broth and inoculation into meat, suggesting stationary phase cells

d. *C. perfringens* grown for 6 hrs at 37°C in chicken gravy prior to freezing, suggesting exponential to late exponential phase cells.
e. Data not used, as cold lethality studies were conducted in water and lab media.
f. Vegetative cells were likely exponentially growing.

3.13.2.2. Analysis of selected studies for lethality at low temperatures

Concentrations of vegetative cells were assumed to decrease exponentially at temperatures lower than T_{min} (Section 3.11.1) during studies of cold lethality. No formal test of this assumption was performed, but all available data appeared to be consistent with it when any effect of initial cold shock was omitted from consideration. The measured concentrations were modeled by

$$\log_{10}(C) = c_c - a_c t + \varepsilon \qquad \textbf{(3.27)}$$

where the terms are

- C the concentration of vegetative cells of *C. perfringens*,
- c_c a constant corresponding to the concentration of cells at time zero (after the effects of any cold shock,
- t the time of storage at the low temperature,
- a_c the rate of decline ($\log_{10}$ reduction/day) of concentration, and
- ε a normally distributed random term.

Parameters (c_c, a_c, and the standard deviation of ε) and their uncertainties were estimated using likelihood methods.[44] Where multiple experiments using the same experimental protocol were reported in the same study, it was assumed that the standard deviation of ε was the same in each such experiment. Where the experimenter(s) performed replicates of experiments and reported only standard deviations for each measurement (rather than the results of each replicate), the variance of ε was estimated as the sum of the reported variance (square of reported standard deviation) and an experiment-wide variance (for the only such study, Taormina *et al.*, 2003, the additional experiment-wide variance was estimated to be zero).

Table 3.29 Summary of rates of decline ($\log_{10}$ reduction/day) of *C. perfringens* concentrations in refrigerated storage.

Source	Temperature	Product	Slope[a] ($\log_{10}$ reduction/day)	SE
Taormina *et al.*, 2003	4.4	Bologna	0.074	0.018
	4.4	Cured chunk ham	0.089	0.032
	4.4	Cured whole ham	0.040	0.012
Barnes *et al*., 1963	-5	Raw beef blocks	0.005	0.001
	-20	Raw beef blocks	0.0015	0.0012
	1	Raw beef blocks	0.041	0.003

[44] The analyses reported here are performed in the file CP_cold_storage.xls accompanying this risk assessment.

	5	Raw beef blocks	0.036	0.006
	10	Raw beef blocks	0.031	0.006
	15	Raw beef blocks	0.037	0.006
Strong and Canada, 1964	-17.7	Chicken Gravy	0.002	0.002
	-17.7	Chicken Gravy	0.010	0.002
	-17.7	Chicken Gravy	0.014	0.002
	-17.7	Chicken Gravy	0.012	0.002
	-17.7	Chicken Gravy	0.010	0.002
Juneja *et al.*, 1994a	8	Cooked ground beef (Anaerobic)	0.039	0.008
	8	Cooked ground beef (Aerobic)	0.025	0.008
	12	Cooked ground beef (Anaerobic)	0.052	0.008
	12	Cooked ground beef (Aerobic)	0.030	0.008
	4	Cooked ground beef (Anaerobic)	0.048	0.008
	4	Cooked ground beef (Aerobic)	0.030	0.008
Juneja *et al*., 1994b	4	Cooked ground beef (Anaerobic)	0.057	0.012
	4	Cooked ground beef (Aerobic)	0.048	0.012
	4	Cooked ground beef (Aerobic)	0.037	0.012
Kalinowski *et al*., 2003	0.6	Cooked cured turkey	0.201	0.058
	0.4	Cooked cured turkey	0.233	0.058
	10	Cooked cured turkey	0.153	0.058
	0.6	Cooked uncured turkey	0.088	0.058
	0.4	Cooked uncured turkey	0.100	0.058
	10	Cooked uncured turkey	0.120	0.058

[a] The slope of the plot of base 10 logarithm of concentration against time.

Table 3.29 and Figure 3-6 summarize the rate at which *C. perfringens* vegetative cell concentrations decline with time in refrigerated storage. There is no apparent variation with temperature above 0 °C (Figure 3-6), nor below 0 °C, nor with any identified characteristics of the food. The data from Kalinowski *et al*. (2003) stand out as higher than others.

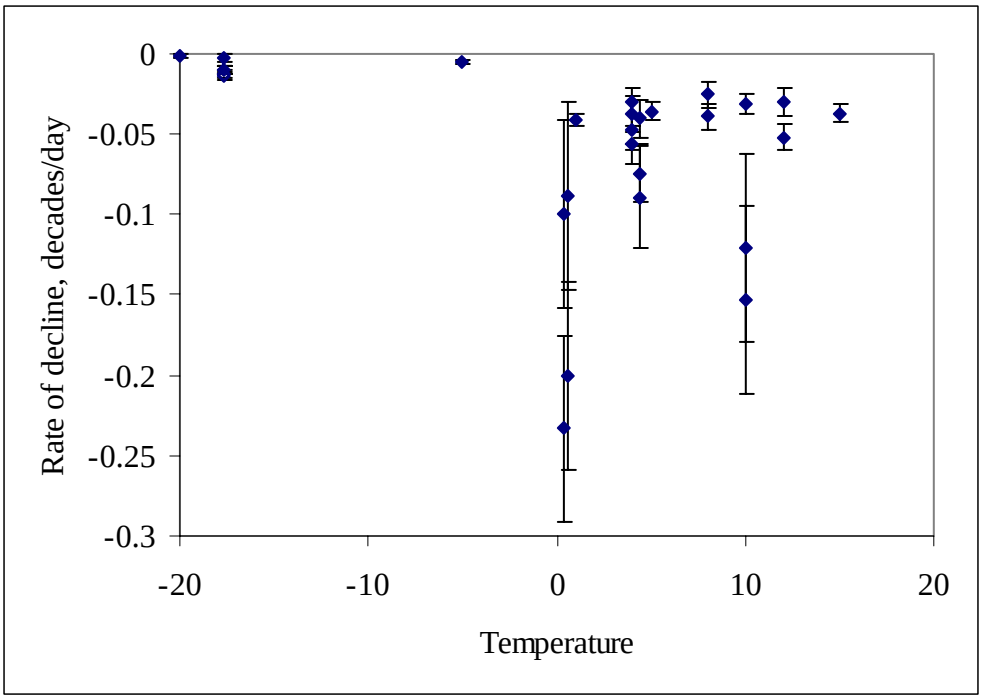

Figure 3-6 Rates of decline of *C. perfringens* concentrations in refrigerated storage (± standard errors).

It is possible that Kalinowski *et al*. (2003) used exponentially growing *C. perfringens*. These authors heat treated 3 mm thin meat samples in pouches to 73.9°C then held the samples at 42 °C for two hours prior to cold shock. Inoculated *C. perfringens* spores would likely have germinated and the product temperature would have equilibrated quickly to 42°C due to the width of the sample. Over two hours at near optimal growth temperature, *C. perfringens* may have entered exponential growth. Additionally, a large differential between the initial temperature and the cold temperature may have increased the lethality of the cold shock (Traci and Duncan, 1974). Kalinowski *et al.* (2003) employed a cold shock differential of 32°C. Substantial initial lethality was observed (and was omitted from the analysis), but the effect on the subsequent decline rate of survivors is not clear.

Despite the discrepancy of the results of Kalinowski *et al*. (2003), it is plausible that similar conditions apply to some RTE and partially cooked foods, so these data were included in the analysis. The variability seen in Figure 3-6 is assumed to be representative of that to be seen in RTE and partially cooked foods, and is modeled by separate lognormal distributions for temperatures above 0 °C and below 0 °C. The parameters for these lognormal distributions, and their uncertainties (which are assumed to be adequately represented by a multinormal distributions[45]), were obtained using likelihood methods from the data of Table 3.29 and are shown in Table 3.30.

[45] The multinormal uncertainty distributions can result in estimates for the standard deviations of the lognormal distribution that are negative. This occurs less than 0.001% of the time for temperatures above 0 °C and less than 4% of the time for temperatures below 0 °C, and in such cases the standard deviation is set to zero. This

Table 3.30 Parameters for the variability and uncertainty distributions for the decline rate of *C. perfringens* cells in refrigerated storage.

Temperatures above zero centigrade

		Natural logarithmic scale	
Arithmetic scale		Mean	SE
Median (log_{10} reduction/day)	0.056	-2.89	0.13
GSD	1.72	0.54	0.11
		Correlation	0.20

Temperatures below zero centigrade

		Natural logarithmic scale	
Arithmetic scale		Mean	SE
Median (log_{10} reduction/day)	0.0089	-4.72	0.17
GSD	1.40	0.33	0.18
		Correlation	-0.21

3.13.2.3. Further assumptions for modeling cold lethality

The measurements on refrigerated storage indicate gradual decline in concentrations of vegetative cells at a temperature as high as 15 °C in one case (Barnes *et al.*, 1963), although the analysis performed in this risk assessment indicates that growth can occur at temperatures down to about 12.5 °C (Section 3.11.3) and growth has been observed as low as 12 °C (Solberg and Elkind, 1970). In this risk assessment, it is assumed that the cutoff point for growth is T_{min} (the value of which is included in the uncertainty analysis, but is close to 12.5 °C, see Sections 3.11.2 and 3.11.3). Below that temperature, *C. perfringens* vegetative cells are assumed to die on average, and above that temperature they are assumed to grow on average.

Spores appear to be not greatly affected by refrigeration and freezing temperatures (Barnes *et al.*, 1963; Solberg and Elkind, 1970; Canada *et al.*, 1964), although some declines in spore concentrations are apparent. In this risk assessment, spores are assumed to be completely unaffected by storage at any temperature encountered in practice, so that the lethality factor l_s in Equation (3.2) is assumed to be unity.

Data used to estimate the effect of freezing temperature require thawing of the meat to measure the *C. perfringens* levels. The combined effect of freezing storage and thawing are therefore reflected in the data analyzed here. It is unknown if the thawing methods used by the researchers

approximation was considered adequate, because the uncertainty in death rates during cold storage contributes so little to the overall uncertainty.

reflect typical thawing methods that might be used by consumers. Moreover, it is unknown whether the freezing methods used in practice will affect *C. perfringens* vegetative cell levels — sufficient cold shock clearly does kill cells, but the degree of cold shock occurring in practical production of RTE and partially cooked foods is not known. Any immediate effects of freezing methods have been eliminated from the analyses performed here, and it is assumed in the risk assessment that they have no effect.

3.13.2.4. Growth during storage

If temperatures in storage rise above T_{min} (Section 3.11.1), vegetative cells will start growing. This process is modeled in the risk assessment by assuming that vegetative cells in RTE and partially cooked foods are ready to enter the exponential phase of growth with no delay period, and applying the growth rates obtained in Section 3.11 for the duration of storage.

3.13.3. Duration and temperature of post-manufacturing storage

The period between manufacturing to consumption of food is assumed to include two storage periods, one between manufacturer and retailer, the second between retailer and final consumption. The times and temperatures of storage vary among RTE and partially cooked products, and is discussed by food category in what follows.[46] Food categories were defined in Section 3.4 and in more detail in Appendix A — briefly the categories are: (1) foods with 2.2%–3% salt in the presence of nitrites; (2) foods unlikely to be reheated before consumption; (3) foods likely to be reheated before immediate consumption; and (4) foods served hot but not necessarily prepared for immediate consumption.

Category 1 and 2 foods.

The FDA/FSIS *Listeria monocytogenes* relative risk assessment (FDA/FSIS, 2003) provides estimated distributions for storage times and temperatures for RTE deli meats and hot dogs stored between their manufacture and arrival at a retail outlet, as well as between the retail outlet and preparation or consumption. These distributions are a combination of estimates from available data and expert opinion. The same distributions are used here where no better information is available, since the previously published distributions have had some public scrutiny.

Between manufacturer and retailer, the storage time for each product is assumed to be uniformly distributed between 10 and 30 days. This is the same assumption used in the *Listeria monocytogenes* risk ranking analysis. No uncertainty is assigned to this variability distribution. The storage temperature for each product, reached at the end of the manufacturing (heating and stabilization), is assumed to be represented by temperatures observed for packaged lunch meat immediately after removal from retail display cases in the Audits International/FDA (1999) survey. The observed empirical distribution is used in this risk assessment (Figure 3-7).[47] The data were reported to 1 Fahrenheit degree and accumulated to counts of measurements at each degree. There is an extreme bias towards even Fahrenheit temperatures in the raw data;

[46] The data and analyses reported in this section are included in the worksheet CP_time_temps.xls accompanying this risk assessment.

[47] The distribution assumed in the Listeria Monocytogenes risk ranking analysis was a uniform distribution between 1 and 5 °C.

however, these data have been used as reported in order to preserve correlations (see below). No uncertainty has been assigned to this distribution.

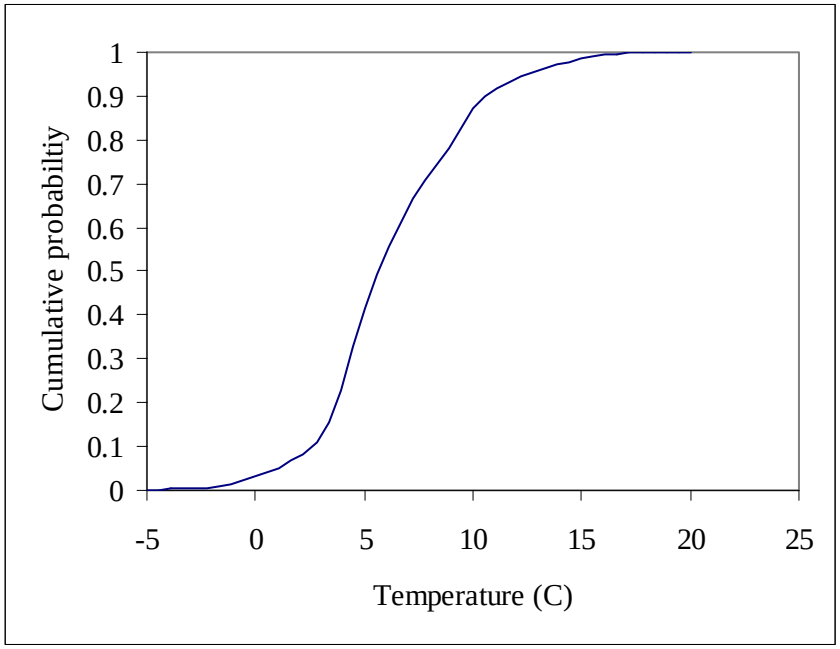

Figure 3-7 Cumulative distribution for the temperature of lunch meat immediately upon removal from its retail display case (based on Audits International/FDA, 1999); these temperatures are assumed to represent storage temperatures for Categories 1 and 2 foods.

For the storage period between retailer and preparation or consumption, data from Audits International/FDA (1999) were used to estimate a distribution of product temperatures, and survey data collected by the American Meat Institute (2001) to estimate a distribution for storage times. Storage temperature is assumed to be represented by the home refrigerator temperatures measured in the Audits International/FDA (1999) survey — the temperature of semi-soft dairy product was measured 24 hours after it was placed in the home refrigerator. This empirical temperature distribution (Figure 3-8) is used as the variability distribution for this risk assessment. Again, the data were reported to 1 Fahrenheit degree and accumulated to counts of measurements at each degree. There is an extreme bias towards even Fahrenheit temperatures in the raw data; however, these data have been used as reported in order to preserve correlations (see below). No uncertainty has been assigned to this variability distribution.

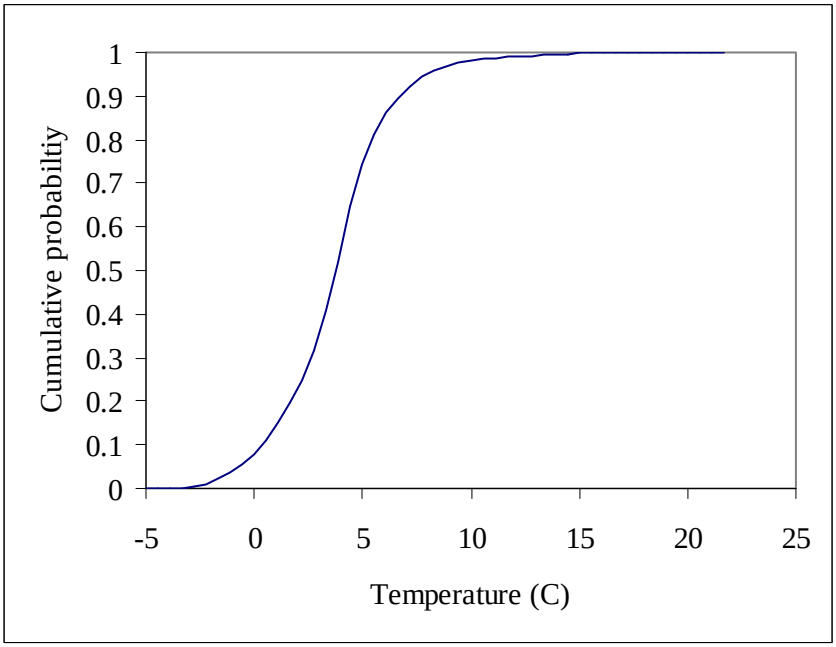

Figure 3-8 Empirical temperature distribution for home refrigeration temperature (based on Audits International/FDA, 1999); assumed representative of post-retail storage temperatures for Categories 1 and 2 foods.

The storage temperatures between manufacturer and retail (pre-retail), and between retail and final preparation or consumption (post-retail), may be correlated (*e.g.* by any effect of ambient temperature on these storage temperatures). In the Audits International/FDA (1999) data (Figure 3-7 and Figure 3-8), there is a slight but significant positive correlation (Pearson correlation coefficient 0.156, $p<0.01$) between the 933 paired measurements available. The 40 unpaired pre-retail and 6 unpaired post-retail measurements are not distinct in distribution from the 933 paired samples ($p > 0.1$ in both cases by both Kolmogorov-Smirnov and Kuiper tests), and their ranges are entirely within those of the paired measurements. To incorporate the correlation, the empirical distributions of paired samples were sampled simultaneously (selecting both measurements at once; so unpaired measurements are not used).

The American Meat Institute survey of 1000 persons (American Meat Institute, 2001) requested information on the average time in storage of prepackaged deli meats and prepackaged hot dogs, reporting numbers of respondents in ranges of periods. The averages so obtained are here assumed to correspond to between-household variation, and the empirical cumulative distribution (Figure 3-9) is used in this risk assessment by interpolating linearly into it.

To incorporate the expected additional intra-household serving-to-serving variation in storage times, a lognormal intra-household distribution was assumed, with a median equal to a random sample from the empirical cumulative distribution (the same as was done in the *Listeria monocytogenes* risk assessment, FDA/FSIS, 2003, although there are no available data to justify the selection of a lognormal distribution here). To estimate the standard deviation of the

lognormal, and its uncertainty, further data obtained from a pilot questionnaire administered to callers to a USDA hotline were assumed representative. A response to a question on the storage time of the last-bought hot dogs was obtained from 29 callers, and the likelihood of the values of storage time that they provided (assuming the distributions just described) used to estimate an uncertainty distribution for the standard deviation of the lognormal. A good approximation to the likelihood was obtained by expressing the uncertainty distribution for the standard deviation (the logarithm of the geometric standard deviation) as a mixture of two normal distributions censored to the left at zero. The probability density for the standard deviation (σ) of the lognormal (specifically, the standard deviation of the underlying normal distribution) is thus estimated as proportional to:

$$\frac{\beta}{q_1}\exp\left(-\frac{1}{2}\left(\frac{\sigma-\sigma_1}{q_1}\right)^2\right)+\frac{1-\beta}{q_2}\exp\left(-\frac{1}{2}\left(\frac{\sigma-\sigma_2}{q_2}\right)^2\right) \qquad \sigma \geq 0 \qquad \textbf{(3.1.28)}$$

where the estimated values are:

σ_1 = 0.0071
σ_1 = 0.4349
q_1 = 0.0769
q_2 = 0.3358
β = 0.3134

No uncertainty is assigned to the resulting distributional estimates for household storage time.

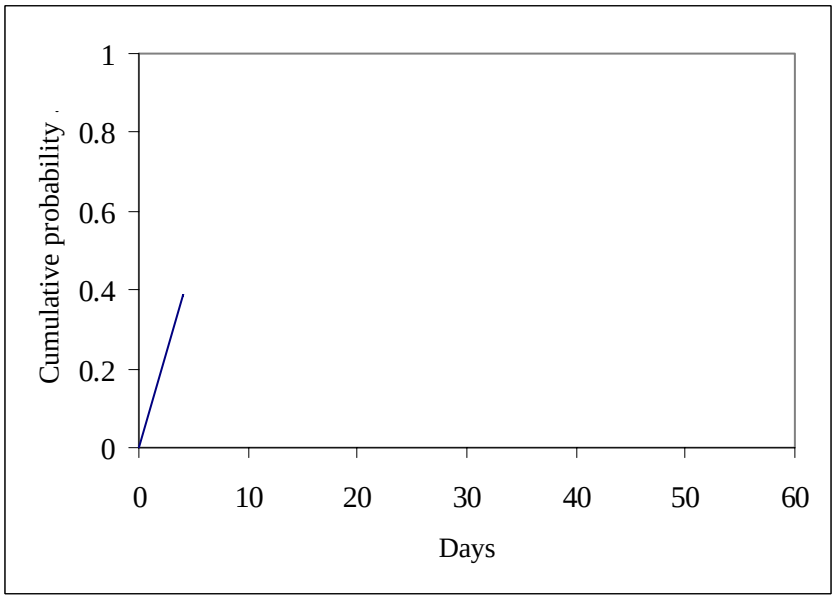

Figure 3-9 Cumulative frequency distribution for average home storage time (American Meat Institute, 2001).

Category 3 and 4 foods.

Category 3 and 4 foods are assumed to be sold frozen. Storage temperatures between manufacturing and retail, and post-retail, were estimated from the Audits International/FDA (1999) survey. It is assumed that the retail storage temperatures of frozen entrées, measured in this survey as the temperature of a frozen entrée immediately after removal from a retail display case, are representative of storage temperatures between manufacturing and retail. For post-retail storage, the temperatures of home freezers, measured in this survey as the temperature of ice cream 24 hours after being placed in the freezer, are assumed to be representative.

The empirical distributions for these temperatures are used in the risk assessment as variability distributions (Figure 3-10 and Figure 3-11). The data were reported to 1 Fahrenheit degree and accumulated to counts of measurements at each degree. There is an extreme bias towards even Fahrenheit temperatures in the raw data; however, these data have been used as reported in order to preserve correlations (see below). No uncertainty has been assigned to these variability distributions.

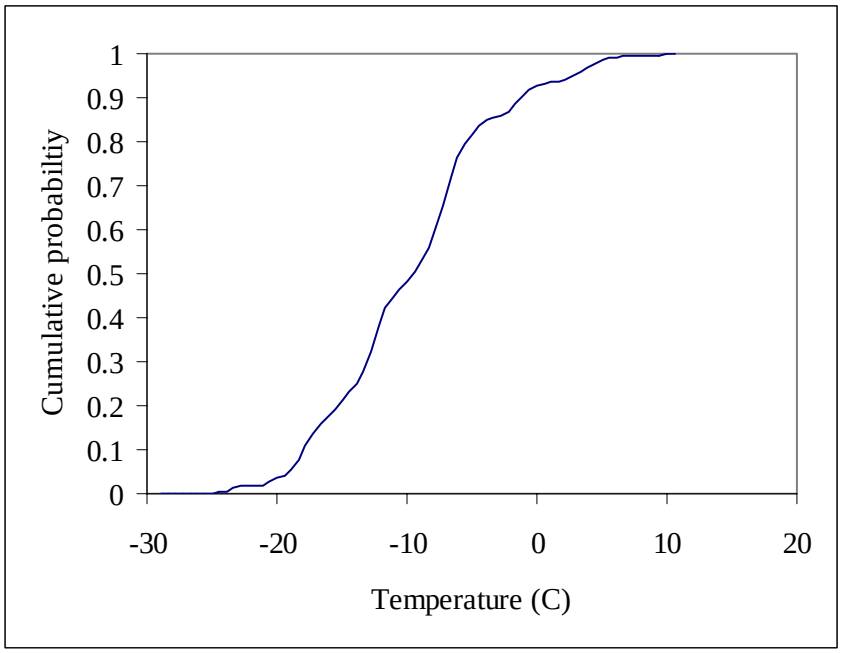

Figure 3-10 Empirical distribution for retail storage temperatures of frozen entrées (based on Audits International/FDA, 1999).

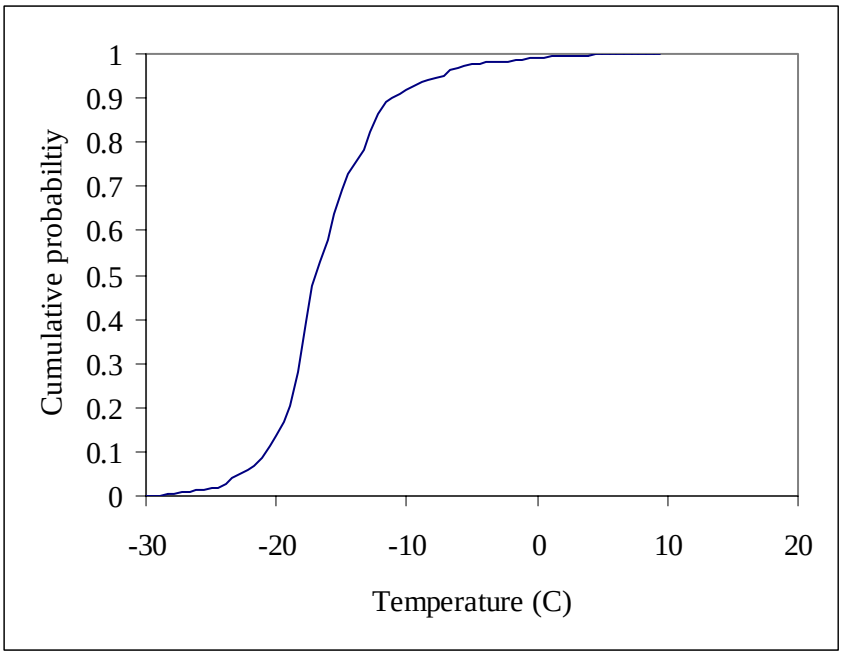

Figure 3-11 Empirical distribution for home freezer temperatures (based on Audits International/FDA, 1999).

The storage temperatures between manufacturer and retail (pre-retail), and post-retail, may be correlated (*e.g.* by any effect of ambient temperature on these storage temperatures). In the Audits International/FDA (1999) data (Figure 3-10 and Figure 3-11), there is a slight but significant positive correlation (Pearson correlation coefficient 0.217, $p<0.01$) between the 888 paired measurements available. The 34 unpaired pre-retail measurements are not distinct in distribution from the 888 paired measurements ($p >0.1$), and their range is entirely within that of the paired measurements. The 52 unpaired post-retail measurements are distinct in distribution ($p < 0.02$ by Kolmogorov-Smirnov test) from the 888 paired measurements (Figure 3-12). To incorporate the correlation, the empirical distributions for paired pre- and post-retail temperatures were sampled simultaneously (selecting both measurements at once). To account for the small difference in the unpaired post-retail measurements, with probability 52/(888+52) the post-retail temperature initially selected is replaced with a random sample from the 52 unpaired post-retail measurements.

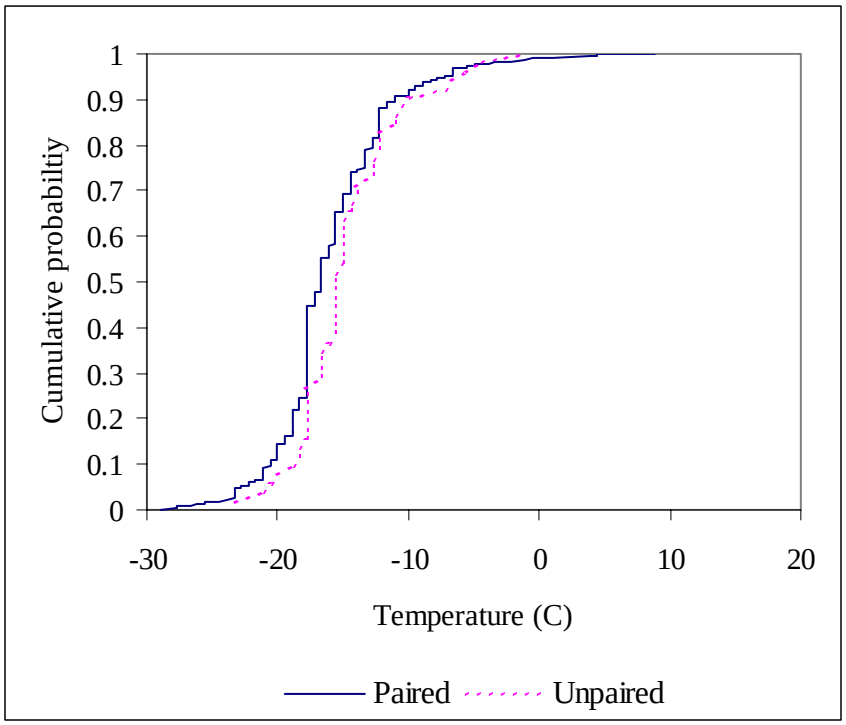

Figure 3-12 Difference between distributions of post-retail storage temperatures for paired (pre- and post-retail) and unpaired (post-retail only) measurements for storage temperatures for Category 3 and 4 foods.

No measurements of duration of storage after manufacture and prior to preparation of foods in categories 3 and 4 have been identified. In their absence, the manufacturing to retail and post-retail times are assumed to be the same as for categories 1 and 2. A sensitivity analysis is performed to evaluate the importance of this assumption.

3.14. Re-heating and hot holding of RTE foods

RTE foods in Categories 3 and 4 are assumed to be reheated before consumption. During such reheating the number of *C. perfringens* vegetative cells may initially increase, so long as the temperature of the food remains below 53.5 °C. As the temperature rises above 53.5 °C, destruction of some to all vegetative cells will occur. The net effect is controlled by the timing and temperature of reheating, with longer times at higher temperatures causing more lethality.

Reheating may also contribute to an increase in vegetative cells if the product is hot-held at too low a temperature after reheating, if the reheated RTE product is cooled from its cooking temperature into a range of temperatures that allow for *C. perfringens* growth. The hazard of reheating is that the holding period after reheating allows for substantial multiplication of any surviving vegetative cells, or of newly germinated spores, before the food is consumed. In this risk assessment, it is assumed that for hot-held foods the reheating is to a sufficiently high temperature that all vegetative cells are killed and spores are activated, so the hazard arises from subsequent holding at lower temperatures allowing the germinated spores to grow.

3.14.1. Evaluation of experimental data on death of *C. perfringens* vegetative cells during heating

The destruction of vegetative cells at high temperatures is generally characterized by D-values. At a fixed temperature, and under specified conditions, the D-value is the length of time taken for the concentration of vegetative cells to decrease by a factor of 10 (1 log10) on the portion of the survival versus time curve that is exponential.[48] Measurements of the destruction of many pathogens at high temperatures demonstrate that over a small temperature range the logarithm of the D-value itself decreases linearly with temperature (and such behavior agrees with simple analogies with chemical reaction rates) if other conditions are held invariant. The rate of decrease of the D-value with temperature is measured by a z-value, the temperature change projected[49] to change the base 10 logarithm of the D-value by unity (that is, to reduce the D-value ten-fold).

Experimental evidence on D-values and z-values for *C. perfringens* vegetative cells were collected and analyzed (Table 3.31).[50] Roy *et al.* (1981) measured D-values and z-values for two strains (NCTC 8238 and 8798) in late exponential growth or early stationary phase after cultivation at four fixed temperatures, or after cultivation at temperatures that were increased linearly with time from 20 °C to 50 °C. In all cases, both cultivation and testing was in autoclaved ground beef (17% or 22% fat). Juneja and Marmer (1998) measured D-values and z-values for a mixture of three strains (NCTC 8238, 8239, and 10240) cultivated at 37°C in fluid thyoglycolate medium (FTM) to stationary phase and then mixed with autoclaved 90% lean ground beef and chicken (with fat poured off while the meat was hot). Smith *et al*. (1981) examined D-values in a heat-resistant strain (S-45) cultivated in FTM to maximum stationary phase and then tested in FTM at fixed temperatures of 60°C and 65.5°C.

Examination of the D-values and their variation with temperature indicated that they could be classified into two classes. The first are those obtained after cultivation of *C. perfringens* vegetative cells at constant temperatures of 37 to 45 °C, followed by determination of D-value at a temperatures 15 °C or more higher than the cultivation temperature, involving a substantial heat shock (Figure 3-13). The second are those obtained after cultivation of *C. perfringens* vegetative cells at temperatures higher than 45 °C or with the temperature increasing at a constant rate before determination of the D-value, so that heat shock was minimized (Figure 3-14).

[48] At short times, there is often a rapid drop in survival before a steady exponential decline; and at later times the curve may "tail" in a non-exponential fashion. The former may be due to the rapid increase in temperature used in some experiments killing some susceptible fraction of the population. The latter may be attributable to some fraction of particularly hardy organisms, especially in cases where multiple strains are tested together.

[49] The actual temperature range used for the measurement may be less than that required to reduce the D-value ten-fold. The z-value is more generally the negative of the inverse of the slope of the log(D-value) versus temperature curve.

[50] All calculations reported in this section were carried out in the workbook CP_D_values.xls accompanying this risk assessment.

Table 3.31 Summary of available data on D-values (in minutes) for *C. perfringens.*

	Temperature, °C							
	55	57	57.5	59	60	61	62.5	65.6
Conditions[a]	D-values in minutes[b]							
Juneja and Marmer, 1998, mixed NCTC 8238, 8239, and 10240								
Lean Beef, cultivation temp. 37 C	21.6		10.2		5.3		1.6	
Turkey, cultivation temp. 37 C	17.5		9.1		4.2		1.3	
Roy *et al*., 1981, NCTC 8238								
Beef, cultivation temp. 37 C		7.3		2.3				
Beef, cultivation temp. 41 C		10.2		3.0				
Beef, cultivation temp. 45 C		17.2		4.1				
Beef, cultivation temp. 49 C				6.9				
Beef, cultivation ramp 4 C/hr				7.6				
Beef, cultivation ramp 6 C/hr	122.0	17.0		11.9	3.7	3.7		
Beef, cultivation ramp 7.5 C/hr				6.8				
Roy *et al*., 1981, NCTC 8798								
Beef, cultivation temp. 37 C		11.0		3.1				
Beef, cultivation temp. 41 C		13.7		4.4				
Beef, cultivation temp. 45 C		24.3		5.2				
Beef, cultivation temp. 49 C				10.6				
Beef, cultivation ramp 4 C/hr				11.0				
Beef, cultivation ramp 6 C/hr	179.0	21.0		8.4		2.3		
Beef, cultivation ramp 7.5 C/hr				7.6				
Smith *et al*., 1981, S-45								
FTM, cultivation temp. 37 C					5.4			0.65

a. cultivation temp.: cultivated at a fixed temperature lower than the test temperature;
cultivation ramp: cultivated in a rising temperature, generally terminating at the test temperature.

b. Geometric means of multiple values where multiple experiments were made under the same condition. The D-value is the length of time taken for the concentration of vegetative cells to decrease by a factor of 10 (see text).

For this risk assessment, these two classifications were used to derive z-values for each situation, which were assumed to apply to microwave cooking (large heat shock) or oven cooking respectively (lesser heat shock). The D-values (from Table 3.31) shown in Figure 3-13 and Figure 3-14 were separately fitted with exponentially declining curves according to the model

$$\log_{10} D_{ij} = \alpha - \beta\left(T_j - T_0\right) + \varepsilon_{ij} + \theta_i \qquad \textbf{(3.29)}$$

where D_{ij} is the geometric mean measured D-value at temperature T_j in experiment i, α and β are parameters (the latter being the inverse of the z-value), T_0 a convenient reference temperature, ε_{ij}

a random experimental error, and θ_i a random fluctuation from experiment to experiment. The random experimental error was assumed to be normal with standard deviation σ, and the random fluctuation was also assumed to be normal with a standard deviation θ. The loglikelihood for the observations is then (up to a constant)

$$J = \sum_i \left\{ -(n_i - 1)\ln\sigma - \frac{1}{2}\ln\left(\sigma^2 + n_i\theta^2\right) + \frac{1}{2\sigma^2}\left[\sum_j s_{ij}^2 - \frac{\left(\theta\sum_j s_{ij}\right)^2}{\sigma^2 + n_i\theta^2} \right] \right\} \qquad \textbf{(3.30)}$$

where $s_{ij} = \log_{10} D_{ij} - \alpha + \beta\left(T_j - T_0\right)$

and n_i is the number of temperatures for which a D-value was measured in experiment i.

The parameters α, β, σ, and θ were estimated by maximizing the expression (3.30), and the uncertainties of α, β, and θ approximated by the usual normal approximation to the likelihood function (with variance-covariance matrix equal to the inverse of the information matrix), treating σ as a nuisance parameter (re-optimizing on σ while computing the information matrix for α, β, and θ). The reference temperature T_0 was selected to make the correlations between the uncertainty estimates for α and β small, to improve the normal approximations for these uncertainties.

Table 3.32 shows maximum likelihood estimates for α, β, and θ for the two situations examined (with substantial heat shock, and with less heat shock), and Table 3.33 summarizes the multinormal uncertainty distributions obtained for these parameters. The maximum likelihood estimate for θ with less heat shock is zero, and it is relatively close to zero (approximately 2.4 standard deviations away) in the case of substantial heat shock. In both cases, in the Monte Carlo analysis, the multinormal distribution is re-sampled until θ is positive.

Table 3.32 Maximum likelihood estimates for the parameters α, β, and θ.

	Substantial heat shock	With less heat shock
α	0.7507	1.0693
β, per °C	0.1585	0.2755
θ	0.0889	0

Table 3.33 Standard deviations (main diagonal) and correlation coefficients (off diagonal) for the parameters α, β, and θ.

	With substantial heat shock		
	α	β, per °C	θ
α	0.0419		
β, per °C	-0.0085	0.0139	
θ	0.0197	0.3787	0.0544
	With less heat shock		
	α	β, per °C	θ
α	0.0331		
β, per °C	0.0195	0.0189	
θ	-0.0016	-0.0035	0.0371

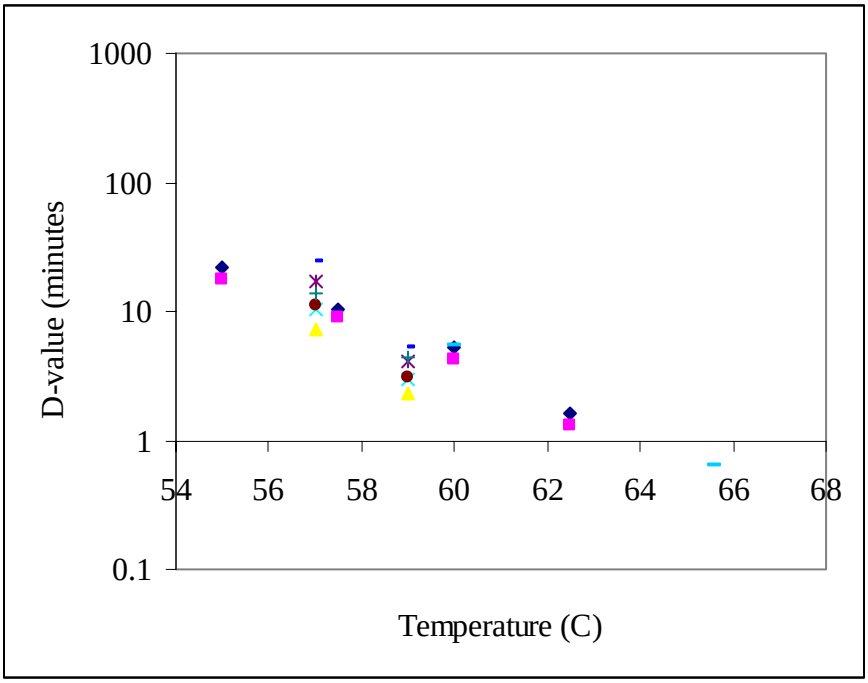

Figure 3-13 D-values where the cells were subjected to substantial heat shock.

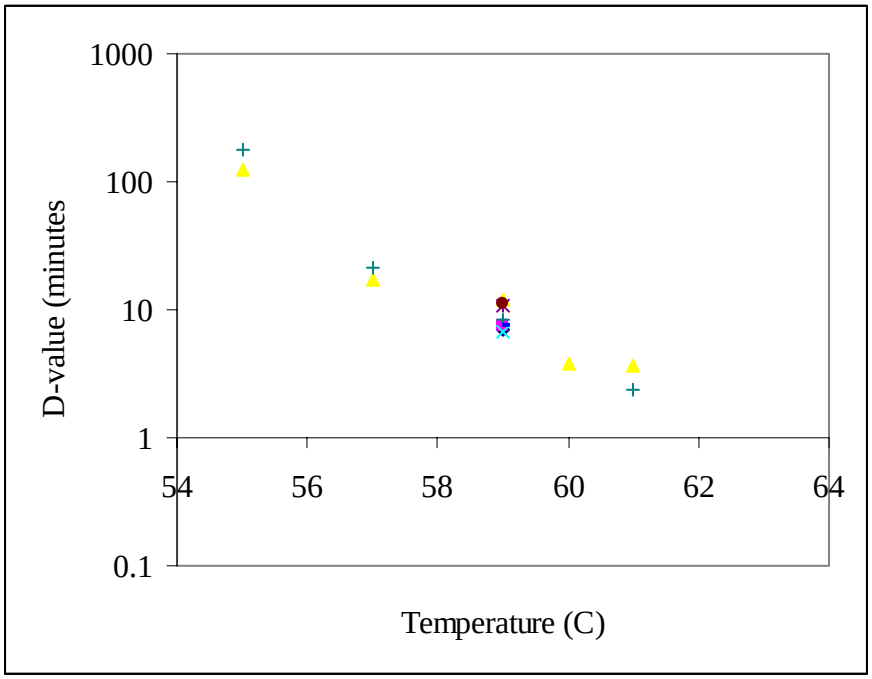

Figure 3-14 D-values obtained under conditions with less heat-shock.

3.14.2. Re-heating times and temperatures.

A total of 3387 cooking temperatures for foods were measured by 608 of 979 participants in a nationwide survey conducted by Audits International/FDA (1999). Those temperature measurements are here assumed representative as a basis for estimating re-heating temperatures for Category 1, 3 and 4 foods. A total of 288 measurements were made on commercially pre-cooked foods, here considered representative of RTE foods, by 224 participants. A performance check on 7% of the participants in the study indicated that temperature measurements were made by 56% immediately after cooking was considered finished, within 1 to 2 minutes by 37%, within 3 to 5 minutes by 5%, and after more than 5 minutes by 2%. Thus some recorded temperatures can be expected to be somewhat lower than the final cooking temperature. The empirical distribution of the measurements on commercially pre-cooked foods (Figure 3-15) shows substantial bunching of recorded measurements at 10 °F intervals (at Fahrenheit temperatures divisible by 10), considered here to be an observational artifact,[51] and a practically uniform distribution with some deviation from uniformity at upper and lower temperatures. In view of the likelihood for measuring temperatures that were lower than final cooking temperature, the bottom tail of the distribution was disregarded; and the upper tail was disregarded as being unimportant in this risk assessment (at the upper temperatures, total destruction of *C. perfringens* vegetative cells would occur very rapidly, Section 3.14.1).[52] The distribution of cooking temperatures used in the risk assessment for all foods in categories 1, 3 (except 3b) and 4, is uniform between 41.5 °C and 87.5 °C (Figure 3-15), values estimated by

[51] The same type of bunching would be expected if cooking was terminated automatically by temperature probes set at such 10 °F intervals, but that is considered less likely.

[52] No sensitivity analysis was performed to evaluate the effects of this treatment of data. Informally, cooking procedures have trivial effects on the results, so these modifications should have negligible effect.

eye to ensure a match with the majority of the empirical distribution. This interpolation of the measurements was preferred to using the empirical distribution itself in order to smooth the measurement artifacts (bunching of observations at 10 °F intervals). The uncertainty of this distribution was considered small enough to ignore, so no uncertainty is assigned to it.

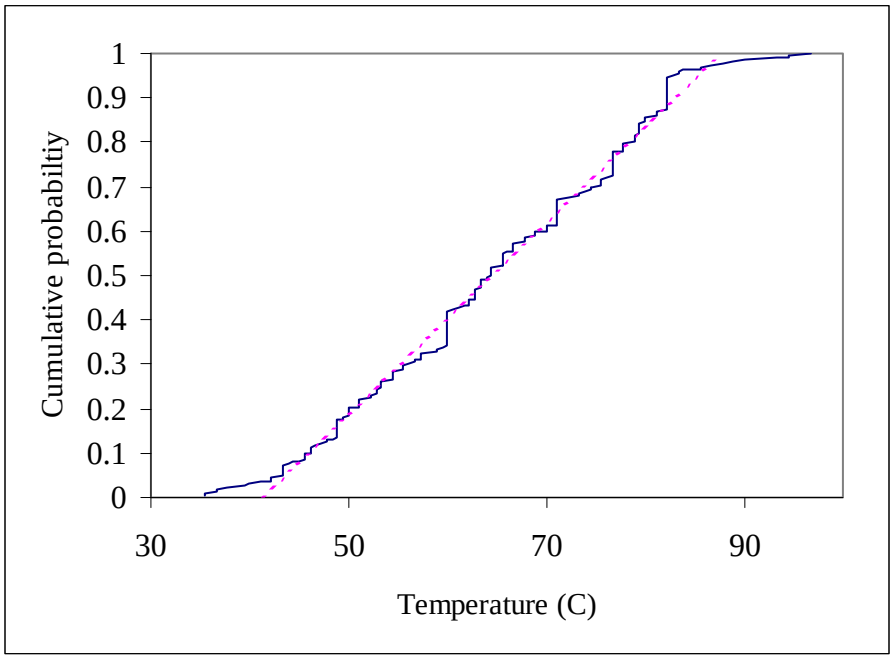

Figure 3-15 Empirical cumulative distribution (black, solid) of measurements of re-heating temperatures for commercially pre-cooked foods, and the uniform distribution used in the risk assessment (mauve, dotted).

Partially cooked foods are assigned to category 3b, and are likely to be heated more thoroughly than RTE foods. The only partially cooked food codes explicitly identified in the CSFII database (USDA, 2000) were described as "chicken patty, fillet, or tenders, breaded, cooked" and "chicken or turkey cake, patty, or croquette." Of the available categories in the Audits International/FDA (1999) survey of cooking temperatures (Beef/Pork/Lamb, Commercially Pre-Cooked, Fish and Seafood, Ground Beef, Poultry, Re-Heated Leftovers, Starch/Dairy/Protein, and Vegetables), the categories Poultry, Ground Beef, and Beef/Lamb/Pork are most likely to represent the temperatures to which partially cooked foods are heated. The distribution of cooking temperatures for these categories considered separately are almost identical (Figure 3-16), and they were combined to represent the cooking temperatures of partially cooked foods. The empirical distribution of the measurements shows substantial bunching of recorded values at 10 °F intervals (at Fahrenheit temperatures divisible by 10), and this bunching is again considered here to be an observational artifact. To remove the effect of such bunching, the empirical distribution was interpolated by a smooth curve that corresponds to a density function initially linearly increasing, and subsequently declining exponentially (Figure 3-17).

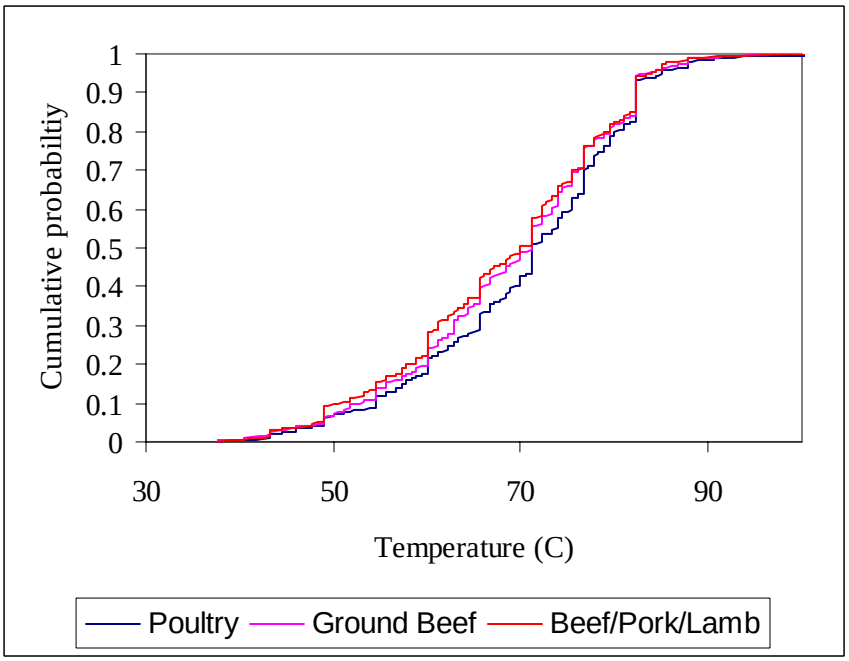

Figure 3-16 Cumulative distributions of cooking temperatures for poultry, ground beef, and beef/pork/lamb categories (Audits International/FDA, 1999).

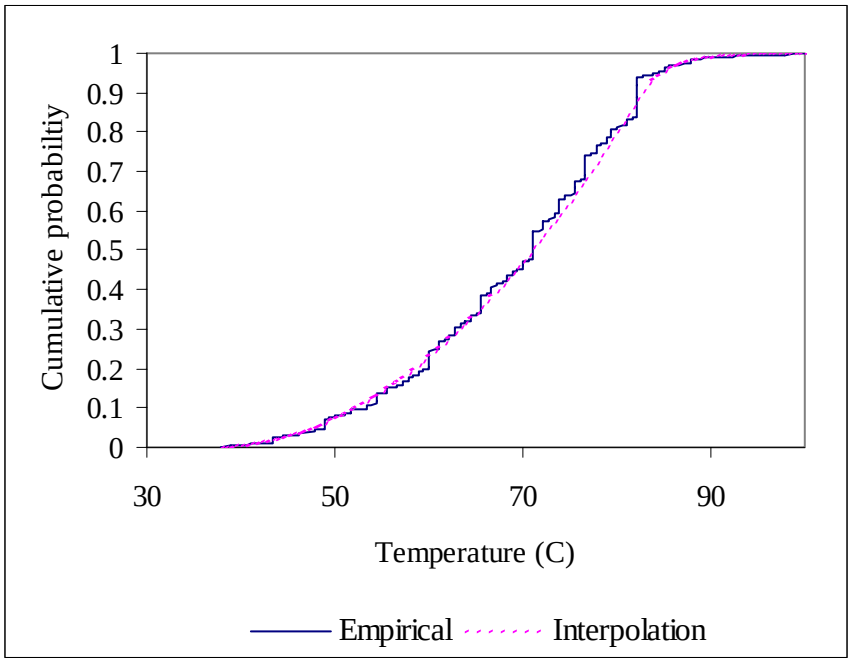

Figure 3-17 Cumulative distribution for cooking temperature for combined Audits International/FDA (1999) categories used to represent partially cooked foods, and the smooth interpolation used in this risk assessment.

The density function used is:

$$\begin{aligned} p(T) &= \alpha(T - T_l) && T_l \le T \le T_u \\ &= \alpha(T_u - T_l)\exp\left(-(T - T_u)/T_f\right) && T \ge T_u \end{aligned} \qquad \textbf{(3.1.31)}$$

$$\text{where} \quad \alpha = \frac{2}{(T_u - T_l)(2T_f + T_u - T_l)}$$

with values:

T_l = 36.73 °C
T_u = 82.22 °C
T_f = 2.941 °C

The uncertainty of this distribution was considered small enough to ignore, so no uncertainty is assigned to it.

Category 3 and 4 foods are all assumed to be reheated before consumption. Some of the category 1 foods, however, will be eaten without re-heating, since some of the foods assigned to Category 1 are customarily eaten cold (*e.g.* ham and cheese sandwich, with lettuce and spread, [not grilled]), while others are occasionally eaten cold (*e.g.* hotdogs, which make up a major fraction of Category 1 RTE foods). The USDA hotline questionnaire obtained some information on eating of hotdogs cold, directly from the package. The available results are ambiguous, although they indicate that between 14 and 46 of 223 persons in the families of the 84 people responding ate hotdogs cold under some (unspecified) circumstances. It is here assumed that 20% of Category 1 foods are eaten without heating, and sensitivity analysis used to evaluate the importance of this assumption.

Independent of the reheat temperature is the time the product takes to reach that temperature, and the time after preparation and before consumption. No survey data were identified that provide information on the times for which foods are heated, or the time before consumption. For the risk assessment it is assumed that 50% of RTE and partially cooked food is heated rapidly, as in a microwave oven, reaching the final temperature in a time that varies from 1 to 10 minutes. This variability is initially modeled as a uniform distribution. The other 50% of RTE and partially cooked foods are assumed to be cooked as in an oven, with cooking times varying from 10 to 30 minutes, again modeled as a uniform distribution. All these parameters are subject to a sensitivity analysis to determine their effect on the risk assessment results. During cooking, temperature of the food is assumed to rise linearly to the final cook temperature at the end of the cooking time. These two assumptions for heating times are categorized as "microwave" and "oven" heating in what follows, but are clearly oversimplifications of what happens during cooking (for example, any method of heating is likely to differentially heat different parts of the food); however, we located no experimental data that would allow taking more complex heating patterns into account. The insensitivity of the results to heating times (Sections 6.6.9 and 6.6.10) suggests that any effects on the risk assessment would be small.

3.14.3. Spore germination during re-heating — the factor g_p

Spores in RTE products may germinate during the reheating step and, therefore, become vegetative cells that can grow during the hot-holding period. In principle, the number of spores that germinate during reheating should be added to the number of vegetative cells that survive

reheating and this total number of vegetative cells would then be capable of multiplying during any hot-holding. For this risk assessment, it was assumed that the number of vegetative cells that survive re-heating prior to hot-holding its zero, so only the number of spores that germinate during re-heating is used.

Individual spores within a population will germinate differently relative to the majority of spores. Specifically, some spores within a population are known as 'superdormant.' These spores tend not to germinate under conditions that normally allow for germination (Gould, 1969). It is possible that the remaining spores following the initial lethality (heating) step at the manufacturing plant will not react to heat treatment as the initial spore population. However, for this risk assessment, it will be assumed all spores react equally to heat treatment. FSIS is unaware of any data that could be used to estimate the population of superdormant spores and the percentage that would germinate due to a second heating. The factor g_p in Equation (3.2) is therefore evaluated using the general analysis of the fraction of spores that germinate on re-heating, in Section 3.9.4.

3.14.4. Hot-holding temperature and time

Many RTE products are consumed immediately after reheating, but category 4 foods are frequently prepared in restaurants or institutions in advance of consumption. Many are frozen products that require reheating before consumption. Such products will be held after reheating for variable times at variable temperatures. Category 1 foods, such as hot dogs, may be similarly handled. The intent of hot-holding is to maintain the product at temperatures above 53.5 °C so that *Clostridium perfringens* growth will not occur; or at least to limit the time product spends in the optimal temperature range for *C. perfringens* growth.

Survey data on temperatures during hot-holding were collected incidentally during an FDA survey on compliance with the 1997 FDA Food Code (FDA, 1997). This survey was national in scope, and designed to be reasonably representative of the industry segments (institutional food service establishments, restaurants, and retail food stores) examined. However, while sampling of the chosen institutions was random within each geographic region that was the responsibility of individual FDA specialists, it was not in proportion to food consumption, so may be biased for the purposes of this risk assessment. Nevertheless, these data are used as though representative on a per-serving basis. A total of 1270 observations of food holding temperatures were recorded during (non-regulatory) evaluation of whether hot-holding temperatures were in or out of compliance with 1997 FDA Food Code requirements for a temperature exceeding 60 °C (140 °F).

The distribution[53] of all 1270 measurements was found to be close to normal (Figure 3-18),[54] with a mean of 63.8 °C (147 °F) and a standard deviation of 13.3 °C (24 °F), but includes many measurements on foods that are not the subject of this risk assessment.

[53] The raw data (censored to remove identifiers) and analyses described in this section are available in the workbook CP_time_temps.xls accompanying this risk assessment.
[54] A formal test rejects normality with high probability.

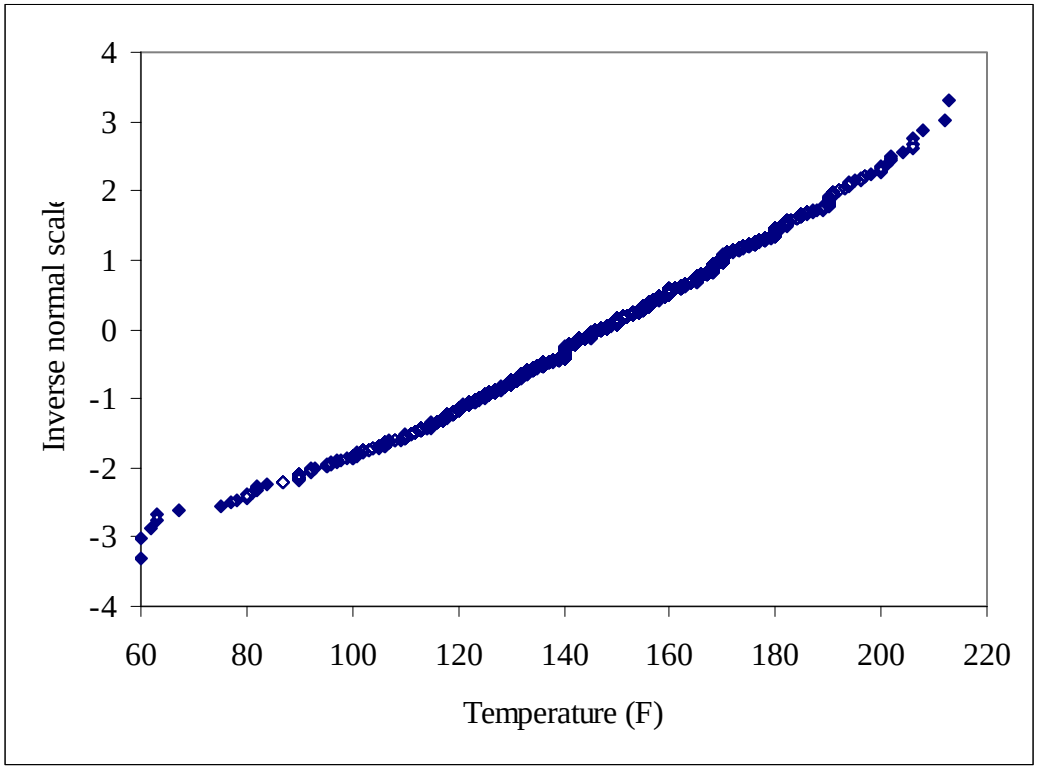

Figure 3-18 Distribution of all hot-holding temperatures found in the FDA survey (FDA, 2000) on a normal scale.

Examination of subsets of the measurements corresponding to potentially meat-containing foods that may have been RTE or partially cooked of categories 1 (n=57), 4a (n=14), 4c (n=27), and 4d (n=72) showed that distributions of measured hot-holding temperatures were roughly consistent with normal.[55] The distributions for categories 4a and 4c were indistinguishable, but those for categories 1, 4a+4c, and 4d were distinct (Figure 3-19)

[55] Formal tests showed marginal normality for category 4a, but the measurements in the other three categories were indistinguishable from normal.

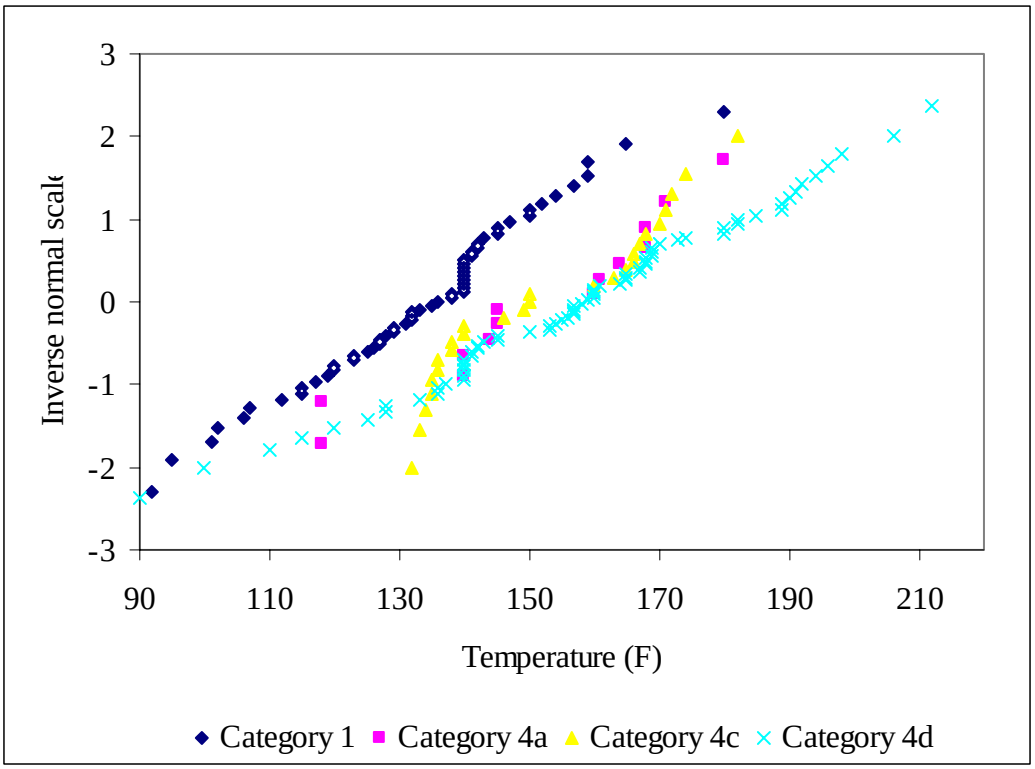

Figure 3-19 Observed distribution of hot-holding temperatures for foods of Categories 1 and 4 (based on FDA, 2000).

Based on these observations, hot-holding temperatures for foods of categories 1, 4a+4c, and 4d were assumed to vary normally with means and standard deviations given in Table 3.34. Uncertainties in these means and standard deviations were estimated using likelihood methods with the assumption that the measurements are representative. The uncertainties are assumed to be normal with parameters also given in Table 3.34 as standard deviations and correlation coefficients.

Table 3.34 Parameters of distributions for hot-holding times. All in °C (except correlations).

	Mean	SD
Category 1	56.27	9.53
	SD (diagonals)/correlation (off axis)	
	1.27	
	0.23	1.03
Category 4a +4c	66.75	9.23
	SD (diagonals)/correlation (off axis)	
	1.45	
	0.27	1.18
Category 4d	69.81	13.34
	SD (diagonals)/correlation (off axis)	
	1.58	
	0.21	1.23

No data on the duration of hot holding was located. The 1997 FDA Food Code calls for a maximum holding time of 4 hours, and holding for substantially longer periods is unlikely since food held for such long periods would likely become unpalatable. Shorter periods of holding seem more likely than longer periods. To evaluate the effect of hot holding period, it is initially assumed that the period varies from 0.5 to 5 hours, with a probability density that decreases linearly to zero at 5 hours. The effect of this assumption is tested by sensitivity analyses.

3.14.5. Growth of *C. perfringens* vegetative cells during hot-holding

Vegetative cells already present in the food, or spores newly germinating during re-heating, may proliferate in hot-held food and present a hazard. For this risk assessment, it is assumed that hot-held food is initially heated sufficiently hot to activate spores and kill all vegetative cells present. Subsequently growth is assumed to proceed as detailed in Section 3.11.

3.15. Numbers of servings

3.15.1. Total number of servings of RTE and partially cooked foods

Two estimates have been made of the total number of servings represented by the foods selected from the CSFII survey (USDA, 2000) for inclusion in this risk assessment, and which contain RTE and partially-cooked foods.

First, the total number of person-days in the 4-year CSFII survey used as a basis for obtaining food serving data is 42,269 (21,662 day 1 samples and 20,607 2-day samples). There are 26,548 food servings in the sub-set of servings that are sampled for the risk assessment. This implies 0.628 servings per person-day. The population of the U.S. is about 281,000,000 (in 2000, U.S. Census Bureau, 2003) so that a country-year is (281,000,000 people × 365.25 days) or

103,000,000,000 person-days. The total estimated servings in the country for one year is then 64,600,000,000.

Second, each survey person has either one or two days worth of food consumption data and a weighting factor to account for variable probabilities that that person would be selected for interview in the survey. The number of food servings reported to be eaten by a sample person (and selected for use in this risk assessment) was divided by the number of days for which that person was surveyed to give the individual's servings per day (of the servings selected in this risk assessment). This value was multiplied by the person's single day sampling weight, all of these values were added together, and the sum was divided by the sum of all the sampling weights to give a weighted average servings per day of 0.677 for the sampled population (again, this refers to the servings selected in this risk assessment). Multiplying this value by the U.S. population (281,000,000, from the 2000 census) and the days per year gives a total national, annual number of servings of foods selected in this risk assessment of 69,600,000,000.

These second estimate is preferred because it uses the weighting factors for inclusion within the sample; and the difference (about 7%) from the first estimate indicates that the relative uncertainty in this number contributes a small fraction of the total uncertainty in the risk assessment.

Some fraction of the foods selected from the CSFII survey will not be RTE or partially cooked. No survey information has been identified that could be used to estimate this fraction. It will be assumed for this risk assessment that 80% of the servings selected (that is, 55.7 billion servings) represent RTE or partially cooked foods. The same fraction is applied to all categories of food.

3.15.2. Fraction of servings that are hot-held

No survey information has been identified that allow estimation of the fraction of RTE and partially cooked food that is hot held after re-heating. For this risk assessment, it is assumed that 1% of Category 1 and 4 servings are so treated.

Appendices for Chapter 3

Appendix 3.1 Fitting gamma concentration distributions to observed counts

Observational studies on concentrations of vegetative cells of *C. perfringens* in meat samples are generally conducted by sampling the meat, homogenizing and diluting the sample, plating the result diluted mixture on suitable agar, incubating under suitable conditions, and counting the resultant colonies of bacterial cells (sometimes with additional safeguards such as confirmation that the colony consists of *C. perfringens*). The procedure is often performed with duplicates of the diluted sample (applying to multiple agar plates), or with replicates of the original meat sample (a second sample put through an identical sequence of homogenization, dilution, and plating), or both. Thus the data available from such sampling consists ultimately of the quantity of meat that was effectively plated, together with a count of the colonies[56] associated with that quantity of meat, which count is taken to equal the number of CFUs in the quantity of meat that was plated.[57]

Suppose the effective quantity of meat plated from a particular sample is m (mass; this may be the sum of the effective quantities applied to multiple plates), and the concentration of viable vegetative cells in the sample is x (CFU per unit mass). The expected number of viable cells plated is then simply mx, and the probability g(r,x,m) to see a particular number r of colonies from that particular sample is just poisson distributed:

$$g(r,x,m) = \frac{(xm)^r \exp(-xm)}{r!} \qquad \textbf{(A3.1.1)}$$

Now if in multiple samples the CFU concentration varies from sample to sample, and the distribution $p(x,a,b)$ of the concentration is gamma distributed:

$$p(x,a,b) = \frac{(x/b)^{a-1} \exp(-x/b)}{b\Gamma(a)} \qquad \textbf{(A3.1.2)}$$

then the probability $P(r,m,a,b)$ to obtain exactly r colonies in any given sample is

$$\begin{aligned} P(r,m,a,b) &= \int_0^\infty dx\, p(x,a,b) g(r,x,m) \\ &= \left(\frac{bm}{1+bm}\right)^r \left(\frac{1}{1+bm}\right)^a \frac{\Gamma(a+r)}{r!\Gamma(a)} \end{aligned} \qquad \textbf{(A3.1.3)}$$

Then for an experiment in which N total samples were measured using a common methodology (same value of m, *i.e.* same sensitivity, for each sample), and exactly k_r of those samples were measured with r colonies of interest (where necessarily $\sum_{r=0}^{\infty} k_r = N$), the loglikelihood J is given by

[56] In some circumstances, particularly with high expected plate counts, plates with zero counts are discarded as being incubation failures.

[57] One could correct for a (fixed) plating inefficiency, but such a correction makes no essential difference to the following discussion. Incorporation of distribution for plating efficiency would be possible, but we have no data to evaluate such a distribution.

$$J = \sum_{r=0}^{\infty} k_r \ln\left(P\left(r,m,a,b\right)N/k_r\right) \qquad \textbf{(A3.1.4)}$$

The normalization adopted here gives $J = 0$ for an exact fit of the probabilities P to the observed fractions k_r/N. Terms with $k_r = 0$ contribute zero to the loglikelihood.

In some cases, the exact values of the r are not known for a given sample, but some information is known. For the data of Kalinowski *et al*. (2003), in one case it was known that 48 colonies were observed on a given sample, of which 5/12 were confirmed as *C. perfringens*. The general case would be that s/S measured colonies, of a total T colonies observed for the sample, are confirmed to be of the type of interest. In that general case, the probability p_r for exactly r colonies of interest is just

$$p_r = \frac{\binom{S}{s}\binom{T-S}{r-s}}{\binom{T}{r}} \qquad \textbf{(A3.1.5)}$$

and the contribution of that particular sample to the loglikelihood may be taken as

$$\ln\left(\sum_{r=s}^{T-S+s} p_r P\left(r,m,a,b\right)\right) \qquad \textbf{(A3.1.6)}$$

(this has no convenient normalization).

For the data of Taormina *et al*. (2003), the published information does not allow an exact specification of the pattern of (r,k_r) pairs, since the published data are consistent with six such patterns. Suppose that there are q such patterns, k^j_r, indexed by j. Then the likelihood for the published result is just

$$\ln\left(\sum_{j=1}^{q}\exp\left(\sum_{r=0}^{\infty} k_r^j \ln\left(P\left(r,m,a,b\right)\right)\right)\right) \qquad \textbf{(A3.1.7)}$$

Again, this has no convenient normalization.

The available data from the studies on raw meat (Section 3.7) varied from study to study. Strong *et al*. (1963) provided only the total number of samples, the number with detections, and the range of estimated concentrations. This allows an approximate calculation of the loglikelihood (approximate[58] since the concentrations are only estimates) by calculating the expected probability for concentrations to be below the bottom of the range of reported concentrations, within that range, and above the end of that range from the gamma distribution (A3.1.2). The probability $P(x_1,x_2)$ for an observation to be within a given range of concentrations x_1 to x_2 is just

$$P\left(x_1,x_2\right) = \int_{x_1}^{x_2} \frac{\left(x/b\right)^{a-1}\exp\left(-x/b\right)}{b\Gamma\left(a\right)}dx = I\left(a,x_2/b\right) - I\left(a,x_1/b\right) \qquad \textbf{(A3.1.8)}$$

where I is the incomplete gamma distribution integral

[58] Approximate also because we are ignoring that the upper end of the concentration range, at least, was not pre-selected but is in fact an order statistic for these data.

$$I(a,x)=\frac{1}{\Gamma(a)}\int_0^x t^{a-1}e^{-x}dx \qquad \textbf{(A3.1.9)}$$

Then the loglikelihood for r observations of concentrations below a detection limit x_1, $n–r$ observations of concentrations in the range from the detection limit to a maximum observed concentration of x_2, and no observations of any higher concentrations, is just

$$r\ln P(0,x_1)+(n-r)\ln P(x_1,x_2) \qquad \textbf{(A3.1.10)}$$

Taormina *et al*. (2003), in addition to reporting the range of concentrations, also reported the mean concentration of those detected. This allows an additional approximate term[59] to be added to the loglikelihood of the form

$$-\ln(\sigma)-0.5\left((m-\mu)/\sigma\right)^2 \qquad \textbf{(A3.1.11)}$$

where m is the observed mean value of the detects, and μ and σ are respectively the expected value of that mean, and its expected standard error, given by

$$\mu=ab\left(I(a+1,x_2/b)-I(a+1,x_1/b)\right)\Big/\left(I(a,x_2/b)-I(a,x_1/b)\right) \qquad \textbf{(A3.1.12)}$$

and

$$\sigma=\left(\left(b^2a(a+1)\left(I(a+2,x_2/b)-I(a+2,x_2/b)\right)\Big/\left(I(a,x_2/b)-I(a,x_2/b)\right)-\mu^2\right)\Big/(n-r)\right)^{1/2} \qquad \textbf{(A3.1.13)}$$

Foster *et al*. (1977) reported numbers of samples within ranges of estimated CFU/g, but in such a way as to allow deduction of the corresponding ranges of observed colony counts. In addition, they reported the mean concentration observed. This allows use of the distribution given in equation (A3.1.3), giving likelihood contributions of the form

$$\left(\sum_r k_r\right)\ln\left(\sum_r P(r,m,a,b)\right) \qquad \textbf{(A3.1.14)}$$

for each range of colony counts, where the sums are over the specific colony counts within that range, and the terms have the same meaning as for equations (A3.1.3) and (A3.1.4) (so in this case only these sums of k_r are known, not the individual k_r). Finally, the mean may be used to give an additional approximate loglikelihood contribution of the form of equation (A3.1.11), where again m is the observed mean concentration, and μ and σ are respectively the expected value of that mean, and its expected standard error. For the distribution given in equation (A3.1.3), these are (assuming a total of N samples)

$$\begin{aligned}\mu&=ab\\ \sigma&=\sqrt{ab(b+1/m)/N}\end{aligned} \qquad \textbf{(A3.1.15)}$$

Estimates for the parameters a and b were obtained by maximizing the likelihood (using the Solver in Excel®). If more than one experiment was fitted simultaneously (*e.g.* with a common parameter), all relevant parameters were estimated simultaneously to maximize the sum of the loglikelihoods, with constraints on the parameters, or relations between them, if necessary. Joint

[59] The approximation is two-fold — a normal approximation for the distribution of the mean, and an approximation induced by the omission of any correlation between the mean estimate and the other information used in the likelihood estimate. Both approximations should be accurate here.

uncertainty distributions for the parameters were obtained by first finding transformations of the parameters such that the individual marginal profile likelihoods for the transformed parameters were approximately quadratic (so that the profile likelihood behaved approximately as a normal distribution). The object was to obtain a parameterization of the loglikelihood in which a (multi-dimensional) quadratic approximation about its maximum value was reasonably accurate over a range extending out several standard deviations, so that the uncertainty distribution approximated the likelihood reasonably closely over as large a range as possible. Empirical investigation of some of the loglikelihoods used in this risk assessment showed that the procedure adopted substantially improved the quadratic approximation (although further improvement was generally possible).

The variance-covariance matrix for the transformed parameter estimates was approximated numerically by inverting an approximation of the information matrix (the matrix of second derivatives with respect to the transformed parameters, evaluated at the maximum likelihood). The second derivative matrix at the maximum likelihood was approximated numerically by making small changes in the transformed parameter values away from the optimum, first one parameter at a time, then in pairs. The resulting changes in loglikelihood were fit in the same sequence as just described to the corresponding quadratic approximation in second derivatives. The sizes of the small changes were generally chosen to approximate the standard deviations of the transformed parameter estimates, so that correlations at relatively large deviations would not be inadvertently omitted. The uncertainty distribution for the transformed parameters was then taken to be a multinormal[60] distribution with the numerically estimated variance-covariance matrix.

[60] The multinormal distribution has a density that is proportional to the exponential of minus a quadratic form in the vector of variates. This distinguishes it from the many other multivariate distributions with normal marginal distributions.

Appendix 3.2 Growth models for *C. perfringens*

A3.2.1 Some background mathematics

Modeling of growth for *C. perfringens* from spores following heat shock has mostly been based on empirical fits to growth curves, with only heuristic connections between the parameters of the models and biological phenomena. Usually what have been used are Gompertz or logistic curves fit to observed counts of CFU density, or more usually to the logarithm of the density, the density including both vegetative cells and any remaining spores that can germinate under the cultivation conditions used for CFU counting (generally different from the growth conditions under test). While such empirical fits to growth curves can provide a very useful summary of the growth to be expected under the conditions tested, extrapolation to other conditions is impeded by the lack of direct connection between model parameters and biological phenomena. The model parameters have to be interpreted in some biologically plausible way in order to make inferences about them under different conditions; and such plausibility arguments are difficult to test without a more rigorous basis for the models.

An approach that may allow more direct inferences of growth under alternative conditions is to explicitly model the biological phenomena involved. The choice of mathematical models is then generally governed by a combination of factors, including incorporation of plausible mathematical representations of the biological processes, and convenience, usually interpreted so that the resulting equations are exactly soluble, easily computed, or have simple structure.

Primary models[61] for bacterial growth at fixed temperature directly attempt to separate the processes of spore germination and vegetative growth. The spore is envisioned as going through some process or set of processes that result in it forming a vegetative cell capable of replication. Before such processes are complete, replication is impossible; after they are complete, replication proceeds at some rate that can be characterized by a growth rate. Replication continues until high vegetative cell densities, at which point some feedback mechanism slows down replication until it stops entirely at a limiting cell density.

The latest models to examine particular and distinguishable processes occurring are of the form (Juneja and Marks, 2002; Huang, 2004):

$$\frac{\partial C_s}{\partial t} = -kC_s$$
$$\frac{\partial C_v}{\partial t} = qkC_s + \mu C_v\left(1 - C_v/C_m\right) \qquad \textbf{(A3.2.1)}$$

where the terms are

C_s	number of viable spores
Cv	number of dividing, vegetative, cells
C_m	maximum number of dividing cells
k	transformation rate of spores (possibly time-dependent)

[61] "Primary" models relate cell density to time at fixed temperature. "Secondary" models then relate the parameters of the primary model to temperature.

μ growth rate for dividing cells (possibly time-dependent)
q the fraction of transformed spores that survive to divide.

Partial derivatives are used to indicate fixed temperature. The boundary condition examined here is that $C_v = 0$, $C_s = C_0$ at $t = 0$. In all cases discussed below, $q = 1$ is selected (Juneja *et al.* 2001 examined $q \neq 1$ to some extent; however, in most cases only those spores that are capable of transforming are ever enumerated, so that all experiments measure only such spores). The first equation represents the conversion of spores to vegetative cells, and the second the replication of vegetative cells.

Strictly speaking, such equations should be written as probabilistic equations (indicating the probabilities for cells to transform from spore to vegetative state, and then the probability for vegetative cells to divide), to account for the granularity of cell densities, especially at low cell densities. Currently, however, cell densities are treated as continuous quantities, with deterministic equations for them, and that is the approach taken here. For large cell densities, the uncertainties induced by such a treatment should be small. For small cell densities, especially during the early stages of growth where there may be only one or a few cells in any volume of interest, reality is likely to be more uncertain than suggested by the solutions of these equations.[62]

For short times (where $C_v << C_m$) the last term in Equation (A3.2.1) (the quadratic term) can be ignored. The first equation in (A3.2.1) is trivially integrated (at fixed temperature) with a single quadrature:

$$C_S = C_0 \exp\left(-K\left(t\right)\right) \qquad \textbf{(A3.2.2)}$$

where C_0 is the initial (at $t = 0$) number of spores, and

$$K\left(t\right) = \int_0^t k\left(s\right) ds \qquad \textbf{(A3.2.3)}$$

so the second equation in (A3.2.1) can be reduced to a Riccati equation:

$$\frac{\partial y}{\partial t} = P + \mu y\left(1 - y\right) \qquad \textbf{(A3.2.4)}$$

where

$$\begin{aligned} y &= C_v / C_m \\ P &= qk\, C_s / C_m \end{aligned} \qquad \textbf{(A3.2.5)}$$

so that $P = P(t)$ and $\mu = \mu(t)$ are known functions of time, and $y = 0$ at $t = 0$.

There is no advantage in writing the first equation of (A3.2.1) in the particular form shown. Indeed, it turns out to be more convenient to write

$$\frac{\partial C_s}{\partial t} = -C_0 g\left(t\right) \quad \text{with} \quad \int_0^\infty \mathrm{g}\left(\mathrm{s}\right) ds = 1 \qquad \textbf{(A3.2.6)}$$

where $g(t)$ is some known function of time. Then

[62] Some of the extra uncertainty induced by the integral number of cells may be captured to some extent by uncertainty analyses applied to experimental data, provided the number of cells used in those experiments is close to the numbers that are important in practice.

$$C_s = C_0\left(1-\int_0^t g(s)ds\right) = C_0\left(1-G(t)\right)$$
$$\text{where} \quad G(t) = \int_0^t g(s)ds \quad \text{so} \quad G(\infty) = 1 \qquad \textbf{(A3.2.7)}$$

This is really equivalent to equation (A3.2.2) — writing $K(t) = -\ln(1-G(t))$ gives the exact equivalence— but it allows choosing the functional form of $g(t)$, hence of P, more easily. The definition of y is unaltered, but P is altered to give

$$y = C_v/C_m$$
$$P(t) = qg(t)C_0/C_m \qquad \textbf{(A3.2.8)}$$

The Riccati equation (A3.2.4) has no known analytic solution, so it is difficult to use. There are various assumptions that went into its derivation, including:

a. The rate of transformation of spores to viable dividing cells is independent of the dividing cell density.
b. The rate of division decreases as the limiting density decreases in a way that is adequately modeled by the term $(1 - y)$. [Replacing the term $(1 - y)$ with a function $F(y)$ that is monotonic increasing on [0,1] and tends to zero as y tends to 1 leads to a more generalized equation; for the homogeneous case ($P = 0$), for example, replacing $(1 - y)$ with $-\ln(y)$ gives a Gompertz curve in place of the logistic — see also Section A3.2.3 below.]

Replacing assumption a. with an equally plausible assumption, that the rate of transformation to vegetative cells is independent of cell density, but that the survival of those vegetative cells decreases quadratically to zero as $y \to 1$, leads to an equation with an analytic solution that is much easier to work with. Thus, replacing Equation (A3.2.4) with

$$\frac{\partial y}{\partial t} = P(1-y)^2 + \mu y(1-y) \qquad \textbf{(A3.2.9)}$$

(which is also a Ricatti equation) gives the analytic (fixed temperature) solution

$$y = \frac{z}{1+z} \qquad \textbf{(A3.2.10)}$$

where

$$z(t) = \exp\left(M(t)\right)\int_0^t P(s)\exp\left(-M(s)\right)ds \qquad \textbf{(A3.2.11)}$$

(which is also the small time approximate solution of (A3.2.4), equivalently the solution of the linearized version of that equation), and

$$M(t) = \int_0^t \mu(s)ds \qquad \textbf{(A3.2.12)}$$

In practical applications, there is likely to be negligible difference between equations (A3.2.4) and (A3.2.9), since spore densities are likely to be substantially smaller than limiting densities for dividing cells. Moreover, equation (A3.2.9) is more convenient to work with, because of the availability of an expression for the analytic solution for all times.

A limited set of modifications to the quadratic in y multiplying P are possible, obtaining other equations that have the solution form (A3.2.10). Thus:

$$\begin{aligned}\frac{\partial y}{\partial t} &= P\left(1+\left(\beta-2\right)y-\left(\beta-1\right)y^2\right)+\mu y\left(1-y\right)\\ &= P\left(1-y\right)\left(1+\left(\beta-1\right)y\right)+\mu y\left(1-y\right)\end{aligned} \qquad \textbf{(A3.2.13)}$$

where β is a constant has a solution of the form (A3.2.10) with

$$z\left(t\right)=\exp\left(M\left(t\right)+\beta R\left(t\right)\right)\int_0^t P\left(s\right)\exp\left(-M\left(s\right)-\beta R\left(s\right)\right)ds \qquad \textbf{(A3.2.14)}$$

where

$$R\left(t\right)=\int_0^t P\left(s\right)ds \qquad \textbf{(A3.2.15)}$$

The value β = 1 gives a particularly simple form, and it is straightforward (although a little less convenient) to perform the analysis below with such a modification. However, the differences between all these equations are of order C_0/C_m, which is negligibly small in current applications.

A3.2.2 Application

Juneja *et al*. (2001) suggested using the linearized version of equations (A3.2.1) (that is, omitting the quadratic term on the right hand side in the second equation) with

$$k\left(t\right)=\lambda t^{\alpha-1} \qquad \textbf{(A3.2.16)}$$

but then specialized to α = 1, corresponding to an exponential for P, and μ = constant. This specialization results in easily computed analytic solutions for z in equation (A3.2.11), and over the exponential growth phase z was used in place of y as an approximate solution. Juneja and Marks (2002) used essentially the same approach. Huang (2004) suggests using equations (A3.2.1), but again with $k(t)$ and μ constant (that is, with α = 1), obtaining the solution using a numerical integrator to cover the full range of growth, including the saturation at large times.

The following discussion is more general, and uses equation (A3.2.9) to allow analytic solutions over the full growth range; and such solutions are negligibly different from those of equation (A3.2.4) for C_0/Cm small. Also, since μ = constant (*i.e*. a constant cell division rate or growth rate at constant temperature) appears to fit all available data, that is also assumed in what follows.

A3.2.2.1 Model 1

A simple generalization of k = constant that also allows analytic solutions for z is

$$k\left(t\right)=a+bt \qquad \textbf{(A3.2.17)}$$

since then

$$z\left(t\right)=\frac{C_0}{C_m}\left(e^{\mu t}-e^{-at-bt^2/2}-\mu\sqrt{\frac{2\pi}{b}}e^{\mu t+\left(a+\mu\right)^2/2b}\left[\Phi\left(\sqrt{b}\left(t+\frac{a+\mu}{b}\right)\right)-\left(\frac{a+\mu}{\sqrt{b}}\right)\right]\right) \qquad \textbf{(A3.2.18)}$$

where Φ is the standard normal integral

$$\Phi(x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{x} e^{-x^2/2} dx \qquad \textbf{(A3.2.19)}$$

(this is g_model_1 in the accompanying workbook; evaluation of z is straightforward except for small values of b).

Applying this model to the data of Huang (2003)[63] leads to strong selection for $a = 0$, matching with the expected biological behavior of germinating spores — that they go through some process that takes non-zero time during germination to the vegetative state in which they can start dividing. Indeed, consideration of this behavior suggests selecting for $k(t)$ a function that allows for a very low or zero initial rate of transformation from spore to vegetative cell. The total number of cells transforming should then increase to a maximum and decrease.[64]

A3.2.2.2 Model 2

To test for such behavior, the model given by equation (A3.2.16) was implemented in the form[65]

$$k(t) = \frac{a}{t_m}\left(\frac{t}{t_m}\right)^a \qquad \textbf{(A3.2.20)}$$

so that

$$P(t) = \frac{C_0}{C_m}\frac{a}{t_m}\left(\frac{t}{t_m}\right)^a \exp\left(-\frac{a}{a+1}\left(\frac{t}{t_m}\right)^{a+1}\right) \qquad \textbf{(A3.2.21)}$$

The form of $k(t)$ is here chosen so that $P(t)$ has a maximum at $t = t_m$, and this maximum has a relative width approximately proportional to $1/a$ for large a. This parameterization was chosen to give some physical meaning to the parameters — t_m is roughly the time it takes for a spore to germinate, and a measures the spread of such times. This physical interpretation also allows an easy modification to account for varying temperatures — see Section A3.2.5 below.[66]

Applying (A3.2.21) in (A3.2.4) to the data of Huang (2003) strongly suggests that a is large. This may be due to either a lack of discrimination in the experimental measurements (quite likely) or because spores germinate almost simultaneously (also possible). Direct testing would require some direct observation of germination of the spores that was not interfered with by the vegetative cells; this may be possible optically.

A3.2.2.3 Model 3

Using the model (A3.2.21) is inconvenient because of the lack of analytic solutions. However, initial efforts indicate that a functional form for $P(t)$ that is similar — with a negligible initial

[63] These models have been applied to other experimental data also, but the discussion here is limited. Practical implementations of the models are available in the workbook CP_fixed_temp.xls accompanying this Risk Characterization.

[64] The transformation rate may keep increasing, but with a finite density of initial cells, the number transforming will decrease again after some time.

[65] There is no connection between the a parameter in this paragraph and that in the last. The symbol is just being re-used.

[66] This model is g_model_2 in the accompanying workbook CP_fixed_temp.xls; there is no analytic solution in terms of well known function, so it is implemented using a 5th order adaptive-step-size Runge-Kutta integrator, which works fairly well.

rate and a peaked shape — should be adequate. The effect of different functional forms for $k(t)$ is easiest to implement using the alternative formulation given at equation (A3.2.6). Further work therefore used equation (A3.2.9), with[67]

$$P(t) = \frac{C_0}{C_m} \frac{1}{t_m \Gamma(a)} \left(\frac{at}{t_m} \right)^a \exp\left(-\frac{at}{t_m} \right) \qquad \textbf{(A3.2.22)}$$

which again has a maximum at $t = t_m$, but the relative width is now about $1/\sqrt{a}$. The advantage of this functional form is that equation (A3.2.11) may then be analytically integrated in terms of standard functions:

$$z(t) = \frac{C_0}{C_m} e^{\mu t} \left(\frac{a}{a + \mu t_m} \right)^{a+1} I\left(a+1, t\left(\mu + a/t_m \right) \right) \qquad \textbf{(A3.2.23)}$$

where I is the incomplete gamma integral

$$I(\alpha, x) = \frac{1}{\Gamma(\alpha)} \int_0^x w^{\alpha-1} e^{-w} dw \qquad \textbf{(A3.2.24)}$$

Provided a is reasonably large, a and t_m have natural interpretations; the latter as an average time to germination of a spore, the former measuring the variation in this time to germination. Using the previous definitions (equations (A3.2.7), (A3.2.8), and (A3.2.10)) gives

$$\begin{aligned} C_s(t) &= C_0 \left(1 - I\left(a+1, at/t_m \right) \right) \\ C_v(t) &= C_m \frac{z(t)}{1 + z(t)} \end{aligned} \qquad \textbf{(A3.2.25)}$$

Fitting this model to the data of Huang (2003, and personal communication) gave MLE values for a that ranged from 55 to (effectively) infinity for individual temperatures, and that were not significantly different for any temperature (p=0.99, likelihood ratio test). The MLE for the joint value was effectively infinity ($>10^5$). With this model also, the product μt_m is temperature independent in these data (p=0.16, likelihood ratio test), as are the initial concentrations (p=0.99, likelihood ratio test), and the maximum concentrations (p=0.49, likelihood ratio test) except at 50°C (where the maximum concentration is substantially lower).

A3.2.3 Connection with usual growth curve fitting techniques

It is interesting to observe that the limit $a \to \infty$ in (A3.2.22) (or in (A3.2.21)) gives a simple connection to the usual *ad hoc* fitting of logistic curves to growth data, and suggests a way of modifying those approaches to give parameters that (may) have biological significance. Taking this limit reduces $P(t)$ to a delta function at t_m

$$P(t) = \frac{C_0}{C_m} \delta(t - t_m) \qquad \textbf{(A3.2.26)}$$

Equations (A3.2.4) or (A3.2.9) may then be analytically integrated. For the usually measured[68] (and usually fitted) quantity $C_s + C_v$, the former gives

[67] There no mathematical connection between the parameters in this paragraph and those in the last, although they have been given the same symbols and represent the same physical quantities.

[68] This assumes that the measurement technique will measure all spores that have started to germinate, and all vegetative cells. It is possible that some of the spores that transform to vegetative cells during measurement would not have so transformed in the original mix — if there is any feedback, for example, as implied by (A3.2.9).

$$\begin{aligned} C_s + C_v &= C_0 && \text{for} \quad t < t_m \\ &= \frac{C_m}{1+\left(C_m/C_0 - 1\right)\exp\left(-\mu\left(t - \ _m\right)\right)} && \text{for} \quad t > t_m \\ &= \frac{C_m}{1+\exp\left(-\mu\left(t-t_m\right)+\ln\left(C_m/\ _0 - 1\right)\right)} \end{aligned} \qquad \textbf{(A3.2.27)}$$

Equation (A3.2.9) gives a minor modification:

$$\begin{aligned} C_s + C_v &= C_0 && \text{for} \quad t < t_m \\ &= \frac{C_m}{1+\left(C_m/C_0\right)\exp\left(-\mu\left(t - \ _m\right)\right)} && \text{for} \quad t > t_m \\ &= \frac{C_m}{1+\exp\left(-\mu\left(t-t_m\right)+\ln\left(C_m/C_0\right)\right)} \end{aligned} \qquad \textbf{(A3.2.28)}$$

(There is a slight mismatch at $t = t_m$ in the second equation, corresponding to some spores not germinating to viable vegetative cells in the presence of other vegetative cells, as implied by equation (A3.2.9) — but they might germinate under the conditions used to measure concentrations, for example if diluted).

The same sort of analysis can give a Gompertz growth curve[69] with a slight modification of equation (A3.2.4). If the growth curve is instead given by

$$\frac{\partial y}{\partial t} = P - \mu y \ln y \qquad \textbf{(A3.2.29)}$$

(which has the same generic shape as equation (A3.2.4)), then the solution with a delta function at $t = t_m$ is

$$\begin{aligned} C_s + C_v &= C_0 && \text{for} \quad t < t_m \\ &= C_m \exp\left(\ln\left(\frac{C_0}{C_m}\right)\exp\left(-\mu\left(t - \ _m\right)\right)\right) && \text{for} \quad t > t_m \\ &= C_m \exp\left(-\exp\left(-\mu\left(t-t_m\right)+\ln\left(\ln\left(C_m/\ _m\right)\right)\right)\right) \end{aligned} \qquad \textbf{(A3.2.30)}$$

Equation (A3.2.29) appears less plausible as a representation of biological processes, in that it presumes that the replication rate of cells at very low cell densities is substantially higher than at the intermediate cell densities where replication rates are generally considered maximal.

A3.2.4 Variation of parameter values with temperature

The growth curves discussed so far are for fixed temperatures. As that fixed temperature is changed, the parameter values also change in a regular way. The variation in values is typically fitted by a secondary model of Ratkowsky form, and that approach is adopted here. Thus the variation of growth rate μ with temperature would usually be given by a model of the form

$$\mu = \mu\left(T\right) = a\left(T - T_{\min}\right)^2\left(1 - \exp\left(b\left(T - T_{\max}\right)\right)\right) \qquad \textbf{(A3.2.31)}$$

where the symbols represent:

[69] This Gompertz curve is for the cell density. However, one usual empirical fitting procedure is to use a Gompertz curve to fit the logarithm of cell density.

T temperature,
T_{min} the minimum temperature below which growth does not occur,
T_{max} the maximum temperature above which growth does not occur,
a a parameter of the model, and
b the second parameter of the model.

This model form is entirely heuristic, designed to represent the shape of the growth-rate versus temperature curve (and the shape of other temperature-dependent functions, such as $1/t_m$) observed empirically for various organisms. However, the (a, b, T_{min}, T_{max}, T) parameterization has several disadvantages:

- The parameters a, b do not relate to any obvious feature of the curve — widely varying combinations of these parameters can give curves that are only slightly different. As a result, estimates of a and b based on data are highly correlated.
- The parameters a, b are implicitly positive. However, imposing positivity on them restricts the range of shapes of the curve — in particular, its maximum cannot be any closer to the minimum temperature T_{min} than 2/3 of the way between T_{min} and T_{max}. Allowing a, b to be simultaneously negative removes this restriction, but the connection between the two possibilities is not smooth (a and b tend to positive infinity, then back from negative infinity, as the maximum temperature goes through the point 2/3 of the way between T_{min} and T_{max}). As a result, estimation procedures for a and b can easily obtain unintended results.

To overcome these disadvantages, but retain the standard shape function, the curve was re-parameterized in terms of x_m, the fractional distance downwards between T_{max} and T_{min} of the maximum of the curve, and A, the maximum value of the curve, in the form:

$$\mu = \mu(T) = A\frac{(1-x)^2\left(1-\exp(-\theta x)\right)}{N} \qquad \textbf{(A3.2.32)}$$

where

$$x = \frac{T_{max}-T}{T_{max}-T_{min}} \quad \text{and} \quad N = N(x_m) = (1-x_m)^2\left(1-\exp\left(-\theta(x_m)x_m\right)\right) \qquad \textbf{(A3.2.33)}$$

and $\theta = \theta(x_m)$ is the unique solution of

$$\exp(\theta x_m) = 1+\theta(1-x_m)/2 \quad \text{for} \quad 0 \le x_m \le 1 \qquad \textbf{(A3.2.34)}$$

(this choice of θ ensures that x_m is the location of the maximum of the curve). With this parameterization, the location of x_m can be varied from 0 to 1 while retaining the form (A3.2.32) for the curve (strictly speaking, at x_m = 1/3, the equation takes on a limiting form since both θ and N vanish at that point, but their ratio is well-defined).