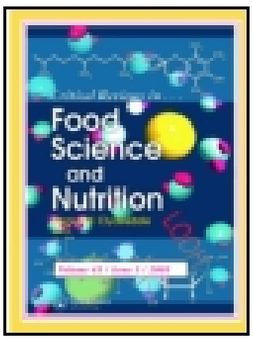

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Thermal processing of live bivalve molluscs for controlling viruses: on the need for a risk-based design

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Abstract

Norovirus (NoV) and Hepatitis A virus (HAV) are the most important viral hazards associated with human illness following consumption of contaminated bivalve molluscs. The effectiveness of the current EU criteria for heat processing of bivalve molluscs (i.e. raising the temperature of the internal mollusc flesh to at least 90°C for a minimum of 90 seconds) was evaluated using predictive microbiology. A HAV thermal inactivation model was developed based on literature data in mollusc matrices during isothermal heat treatment. Application of the developed model demonstrated that the 90°C-90 s requirement may lead to significantly different virus inactivation depending on the commercial process design. This shows the need for the establishment of a Performance Criterion for bivalve molluscs heat processing which will assure a common specified level of consumer protection. A risk-based approach is described that allows for an effective processing design providing a more transparent and objective relation between the thermal processing targets and public health. Model simulations demonstrate that the F-value is a more appropriate Process Criterion than a single time-temperature combination since it enables the food business operators to design a process that is compliant with the safety requirements while at the same time achieving a desired product quality.

Keywords

bivalve molluscs, Hepatitis A virus, Norovirus, heat treatment

Introduction

Bivalve molluscs are filter-feeding marine and freshwater animals which may be consumed as food. Species consumed include mussels, oysters, clams, cockles and scallops, which may be either farmed or harvested. EU annual production of molluscs is around 830,000 tonnes, while total consumption is above 1.3 million tonnes, indicating reliance on extra-EU importation for approximately a third of EU consumption. EU mollusc consumption is around 2.7 kg per capita per year on average or approximately 10% of seafood consumption (EUMOFA, 2016).

In their filter-feeding activity bivalve molluscs may process large amounts of seawater and accumulate a wide variety of microorganisms, potentially including human pathogens. These may be naturally occurring (such as certain environmental *Vibrio* species) or associated with human faecal pollution, such as human enteric viruses. Although many different enteric viruses are present in human and animal faeces and can contaminate bivalve molluscs, Norovirus (NoV) and Hepatitis A virus (HAV) are the most important viral hazards associated with human illness following consumption of contaminated bivalve molluscs (Lees, 2000; Potasman et al., 2002; Bellou et al., 2013). The World Health Organization (WHO) concluded that the virus-commodity combinations of highest priority for risk managers are NoV and HAV in molluscs, fresh produce and prepared foods (WHO, 2008). The European Food Safety Authority (EFSA) similarly concluded that the key viruses of concern for bivalve molluscs were NoV and HAV contamination arising from human faecal pollution of mollusc production areas. Effective control strategies for these viruses need to focus on prevention of contamination, which is to occur primarily at the pre-harvest level for bivalve molluscs. There are currently no effective post-

harvest control options, except sufficient heat treatment, to eliminate the public health risk from viral contamination of bivalves (EFSA BIOHAZ Panel, 2011).

A recent extensive review on reported prevalence and concentration of these viruses in bivalve molluscs (EFSA BIOHAZ Panel, 2015) showed that the reported prevalence of NoV in studies of both consignments on the market and production areas varies significantly and in some cases this prevalence can exceed 70%. Titres of NoV RNA in mollusc digestive tissue are typically quite low (< 100 copies of GI+GII genome per gram), with levels above 10,000 genome copies per gram occurring in < 15% of samples. The highest levels of NoV reported are typically in the range of 10^4 to 10^5 genome copies per gram. Contaminated samples exceeding these levels have not been reported from commercial production areas in the EU. The prevalence of HAV displays a high degree of geographic variability, ranging from 0% to 43%. Prevalence of HAV in molluscs from EU commercial areas seems to reflect the endemicity levels of HAV in EU countries (Gossner et al., 2015) and a discharge of HAV particles into the environment that is temporally discontinuous (Kokkinos et al., 2010; Hellmér et al., 2014; Gossner et al., 2015; Polo et al., 2015). Quantitative data for HAV contamination of shellfish are scarce. However, titres are generally low with levels above 10,000 genome copies per gram reported on average in < 3% of samples and the highest reported values in the range of 10^3 to 10^4 genome copies per gram of digestive tissue (EFSA BIOHAZ Panel, 2015).

EU food legislation (Regulation (EC) 854/2004) requires bivalve molluscs production areas to be 'classified' according to levels of *Escherichia coli*, as a faecal pollution indicator, contained within their flesh and intravalvular liquid. This sanitary classification determines the degree of post-harvest processing required before the products can be placed on the market for human

consumption. Class A molluscs may be placed on the market directly for human consumption without any purification or processing whilst molluscs from Class B and Class C areas must be relayed or depurated prior to placing on the market. As an alternative to those purification options, molluscs from Class B and Class C areas may be heat-treated using one of the approved methods prescribed in EU legislation (Regulation (EC) 853/2004) to eliminate pathogenic micro-organisms. The permitted treatment methods are:

a) sterilisation in hermetically sealed containers;

b) heat treatments involving:

-- (i) immersion in boiling water for the period required to raise the internal temperature of the mollusc flesh to not less than 90°C and maintenance of this minimum temperature for a period of not less than 90 seconds (s);

-- (ii) cooking for 3 to 5 minutes (min) in an enclosed space where the temperature is between 120 and 160°C and the pressure is between 2 and 5 kg/cm^2, followed by shelling and freezing of the flesh to a core temperature of --20°C;

-- (iii) steaming under pressure in an enclosed space satisfying the requirements relating to cooking time and the internal temperature of the mollusc flesh mentioned under (i). A validated methodology must be used. Procedures based on the Hazard Analysis and Critical Control Point (HACCP) principles must be in place to verify the uniform distribution of heat.

Among the above heat treatments, options a and b(ii) are considered adequate to eliminate viral infectivity in molluscs. Options b(i) and b(iii) are alternative approaches for which a Process Criterion (PrC) of a time-temperature combination (not less than 90°C for a period of not less

than 90 s) will not result in a single well-defined level of virus inactivation. Indeed, in commercial processing the rates of temperature increase during heating, and decrease during cooling, (therefore before and after the period of 90°C for 90 s) can vary among processes with different design. Thus two processes that are both compliant with the 90°C for 90 s criterion may have a significantly different effect on overall virus inactivation. In addition, due to the high variability in the levels of virus contamination in raw/live molluscs, the relation between the above PrC and the degree of public health protection afforded may be variable. Although the criteria may deliver variable degrees of virus inactivation, review of the available epidemiological evidence does suggest that these requirements have been effective in protecting public health. There are no reported human outbreaks of infectious illness associated with bivalve molluscs commercially processed accordingly to these legislative requirements (EFSA BIOHAZ Panel, 2015). However, challenges exist in the achievement of the above time-temperature requirements whilst maintaining product quality since the potentially variable nature of the heat treatment delivered can cause overcooking of the product and affect consumer acceptance.

The objective of this paper is to evaluate the effect of the current EU requirements for bivalve molluscs thermal processing on the inactivation of the key viruses HAV and NoV using predictive microbiology tools. Furthermore, a risk-based approach for establishment of thermal processing criteria is proposed which relates process controls to the degree of public health protection desired. The above approach provides flexibility in thermal processing design and allows food business operators (FBOs) to meet targets for both safety and quality of bivalve molluscs.

HAV and NoV inactivation during bivalve molluscs thermal processing

Initially a literature search was performed (details can be found in EFSA BIOHAZ Panel, 2015) to establish the available experimental data on heat inactivation of the key viral hazards HAV and NoV in the bivalve mollusc matrix. Since an *in vitro* cultivation system for human NoV has been lacking until recently (Ettayebi et al. 2016), experimental data are only available based on PCR which is unreliable as an indicator of residual viral infectivity following heat treatment and therefore known to underestimate efficacy of heat inactivation (Croci et al., 2012). Therefore data was only considered for culturable NoV surrogates such as murine NoV, feline calicivirus (FCV) or F-specific RNA (FRNA) bacteriophage. Since inactivation data based on culture was available for HAV and studies have shown that it is more heat resistant than NoV surrogates (Sow et al., 2011), modelling focussed on this pathogen. Retrieved data for HAV were further screened for their utility for modelling and in particular the use of isothermal (above 50°C) experimental conditions and the availability of sufficient data points. A summary of the retrieved HAV studies utilised for modelling are set out in Table 1 and the NoV surrogate studies utilised for comparative purposes in Table 2. Examination of the outputs of these studies shows that the heating temperature is the major factor affecting the D-value, the time for a one-log reduction of the virus. However, for the same heating temperature, significant variations in the D-values are observed among different studies. This can be attributed to the additional factors which may affect viral inactivation such as virus strain, food matrix and heating system (Bozkurt, 2014). Heat inactivation may differ widely between different food types due to matrix effects on virus resistance or due to differences in heat transfer. The mechanisms of thermal inactivation may include denaturation of viral proteins, as well as disassembly of virus particles into non-

infectious viral subunits and single proteins (Song et al., 2010). The mode of action may depend on the temperature and thus different mechanisms may be important at different temperatures. When quantifying the effect of thermal processing on virus inactivation the uncertainty related to all above factors should be taken into account.

Depending on the process design, the temperature achieved in mollusc meat in commercial thermal processing can vary significantly. In addition, commercial processes, in practice, include a heating and a cooling step resulting in a dynamic temperature profile. Thus, to evaluate the overall inactivation of viruses during heat treatment of bivalve molluscs the use of predictive modelling is required. A predictive model can be used to estimate overall viral reductions for any time-temperature combinations and also provide quantitative information on uncertainty. In the present study a predictive model was developed based on HAV data (Table 1) since NoV surrogates may not be representative for human NoV. In addition, HAV is recognised as a particularly robust virus and has been found to be more heat resistant in comparative studies than NoV surrogates (Bozkurt et al 2014a, Bozkurt et al 2014b, Sow et al., 2011). Inactivation data for NoV surrogates (Table 2) was subsequently compared with the predictions of the developed predictive model for HAV.

Predictive modelling HAV thermal inactivation

D-values of HAV thermal inactivation in bivalve molluscs (Table 1) in temperatures ranging from 56 to 100°C were modeled as a function of temperature using the following transformation of Bigelow model.

$$\log D_T = \log D_{ref} + \frac{T_{ref} - T}{z}$$

where T_{ref} is an arbitrary reference temperature (90°C is the temperature selected in the assessment), D_{ref} is the D-value at the reference temperature, T is temperature (in°C) and z is the temperature difference required to achieve a one-log change of the D-value. An analysis employing linear mixed effect models, containing temperature as a fixed effect and random effects to include studies potential heterogeneity, indicated that there was study heterogeneity, and that a model considering studies random effects on both the intercept and the slope described data best (Likelihood Ratio Test (LRT) = 6.57, degree of freedom (df) = 2, $p < 0.037$). Figure 1 illustrates the overall best fit of the mixed model, and the estimated confidence (CI) and prediction intervals (PI) for log D-values. The CI illustrates the variability around the mean log D at the different temperatures and the PI includes the variability around the mean as well as the variability across studies. Overall, for all studies and based on the linear regression shown in Figure 1, the estimated mean log D_{90} was −0.048 and z was 27.5°C. Thus it takes on average 0.9 min to reduce the HAV population by one log unit at 90°C. Further, to obtain a one log change in this inactivation rate would require an average temperature change of 27.5°C. As shown in Figure 1, the 95% PI of the model is similar in shape but wider than the CI. The 95% CI was estimated for the model parameters and the estimated z-values ranged from 13.6 to 41.3°C whereas the D_{90}-values ranged from 0.6 to 1.3 min.

The HAV thermal inactivation model was further validated against HAV inactivation data in whole bivalve molluscs under non-isothermal temperature conditions using data from the studies of Millard et al. (1987), Harlow et al. (2011) and Hewitt and Greening (2006). The results of the validation are presented in Figures 2 (a-f) through a comparison between the observed HAV inactivation data and the prediction of the HAV thermal inactivation model including upper and

lower prediction limits. As shown in the figures mean predictions of the model generally under-predicted HAV inactivation, i.e. the observed inactivation was faster than predicted by the model. The predictions based on the lower 95th PI variably under- or over-predicted the observed non-isothermal inactivation.

To evaluate the ability of the data for HAV thermal inactivation to predict also NoV inactivation, the relationship between log D and temperature as developed for HAV was compared with observations for the NoV surrogates murine NoV and FCV inactivation in bivalve molluscs (data from Table 2). As shown in Figure 3, most of the data points for the surrogate viruses are below or close to the fitted line. The data above the fitted line represent murine NoV inactivation in abalone which, although a mollusc species and therefore a valid tissue model, is not a filter-feeding bivalve mollusc. The limited data suggest that under the conditions and matrices studied, HAV is generally more heat tolerant than surrogate viruses (higher log D), and therefore that the HAV thermal inactivation model is conservative for describing inactivation of NoV surrogates in bivalve molluscs.

Evaluating viral inactivation under current EU requirements

Current EU criteria for bivalve molluscs require raising internal mollusc meat temperatures to a minimum temperature of 90°C and maintaining this for a minimum of 90 s. Considering that the rates of temperature increase during heating and decrease during cooling can vary among commercial processes with different design, differing processes that are compliant with the above requirement may have significantly different effects on virus inactivation due to different heat-up and cool-down durations. For example, Figure 4a shows three hypothetical time--temperature profiles which are all in compliance with the 90°C for 90 s process, but differ in

relation to the rates of temperature increase during heating and decrease during cooling. Profile 1 represents the 'notional' heat treatment process in which temperature is increased instantaneously to the target value during heating and decreased instantaneously during cooling. Profiles 2 and 3 represent time--temperature scenarios with high and low rate of temperature increase and decrease during heating and cooling, respectively. The HAV thermal inactivation model was used to predict the HAV inactivation by applying the heat process according to the above three profiles. As shown in Figure 4b the predicted mean reduction of HAV was 1.67, 2.92 and 4.13 log plaque-forming units (PFU)/g for profiles 1, 2 and 3, respectively. These results illustrate that a '90°C for 90 s' criterion for heating process of bivalve molluscs may lead to significantly different HAV inactivation depending on the commercial process design. Thus, the current EU requirements for heat treatment of bivalve molluscs may result in different degrees of stringency, depending on commercial plant design, rather than a specified level of consumer protection.

A risk-based approach for derivation of Food Safety Objectives for heat processed bivalve molluscs

In the context of risk-based food safety management, the concepts of the Appropriate Level of Protection (ALOP), Food Safety Objective (FSO) and Performance Criteria (PC) (CAC, 2007) are applied to establish a link between targets in the food processing and public health outcomes. The use of Quantitative Microbiological Risk Assessment (QMRA) enables a transparent and objective relation between the PC and FSO or ALOP to be developed.

Figure 5 presents the structure of a risk-based design for thermal processing of foods. Once an ALOP/FSO has been defined by the risk managers, a PC for the thermal processing step can be

established via risk assessment. Table 3 shows the data and the structure of a simple QMRA model developed as a case study to illustrate the relation amongst different PCs for the reduction of HAV during heat treatment of bivalve molluscs and the risk at the time of consumption. The model used data on prevalence and concentration of HAV from a study in one EU Member State (MS) in which sampling was performed in production areas impacted by a community outbreak of HAV and therefore reflects a higher risk scenario for the EU. Using the above QMRA model the effect of various scenarios for the total reduction of HAV during heat treatment (from 0 to 5 logs) on the prevalence and concentration of HAV in mussels at the time of consumption and the risk of infection per serving were evaluated by running a Monte Carlo simulation of the model with 10,000 iterations. The effect of the tested scenarios on the prevalence and concentration of HAV per mussel servings is presented in Table 4. The table provides information on the relation between different FSO´s for HAV in bivalve molluscs, and possible PC´s during heat treatment. For example, meeting a FSO of <100 HAV genome copies per mollusc serving would require a PC of 3 logs of reduction during heat treatment while for a FSO of <10 HAV genome copies per serving the required PC is a 5 log reduction of HAV, with the HAV concentration used in this example.

Figure 6 presents a summary trend graph of the effect of the total reduction of HAV during heat treatment on the HAV concentration per serving assuming the initial concentration distribution in Table 4. Using a dose-response relationship for HAV (Table 3) the concentration at the time of consumption can be translated to probability of infection. Detailed data on the average and the percentiles of the risk of HAV infection per serving of mussels are shown in Table 5. Without heat treatment, the average predicted risk for this scenario is about 1 infection per 100 servings.

For heat treatment resulting in one log reduction of HAV, the average predicted risk would be about one infection per 1,000 servings. For 4 logs reduction of HAV during heat treatment the average predicted risk was about one infection per 1,000,000 servings. The trend of the effect is shown in Figure 7 where the effect of HAV reduction during heat treatment on the relative risk of HAV infection is presented. In general, a one log decrease in HAV concentration during heat treatment resulted in a corresponding one log decrease in the probability of HAV infection per serving. This HAV QMRA model example illustrates in quantitative terms the impact of different PC´s for a heat treatment on the final risk for consumers. However, effective establishment of a PC based on a predefined ALOP (for example a specified acceptable risk of consumer infection per serving of mussels) or a FSO (for example a maximum level of virus contamination following heat treatment), requires a full quantitative risk assessment approach which takes into account the most influential factors affecting risk including other important hazards (i.e. NoV), differences between EU MSs, seasonality of contamination, herd immunity, viral strain-types and virulence, relation between infection/illness etc.

Establishing a process criterion for heat treatment of bivalve molluscs

A PC can be translated to a Process Criterion (PrC) using the HAV thermal inactivation model. As shown in the previous sections, however, the current 90°C for 90 s criterion is not the most appropriate 'Process Criterion' since it may lead to different reductions of HAV depending on process design. A more appropriate method of stipulating a PrC for bivalve molluscs heat treatment processes would be an F-value which is the fundamental concept in thermal process design and evaluation (Stoforos and Taoukis, 2006). The F-value of a non-isothermal heat process is the equivalent processing time of a hypothetical isothermal process at a reference

temperature. Equivalent means that the process produces the same effect (in terms of microbial/viral inactivation) as the actual thermal process.

The establishment of an F-value at a reference temperature as a 'process criterion' requires the definition of the desired total reduction of HAV which can be considered as a 'performance criterion' of the bivalve molluscs heat process. Using the estimated D_{ref} and z-values for HAV inactivation, the F_{90} values for different PC of HAV inactivation during bivalve molluscs heat processing were calculated and presented in Table 6.

When a PrC has been established based on a PC, any heat process of bivalve molluscs should be evaluated for its compliance to this criterion. A heat process is in compliance with a PrC when the following condition is met:

$$\frac{F_{90}\big|_{process}}{F_{90}\big|_{required}} \geq 1$$

The F-value of a heat process can be estimated with numerical integration of the above equation. For example the F_{90} of the heat process with a hypothetical time--temperature profile shown in Figure 8 is 179 s. Based on Table 6 this process is in compliance with the PrC if the selected PC is equal or less to 3 log reductions of HAV (best fit) and non-compliant if the PC is higher.

Conclusions

The current EU requirements for heat treatment of bivalve mollusc ('90°C for 90 s') may lead to significantly different virus inactivation depending on the commercial process and therefore does not assure a common specified level of consumer protection. The use of a risk-based approach as described in the present paper allows for an effective processing design providing a transparent and objective relation between the thermal processing targets and public health. The F-value is a

more appropriate PrC than a single time-temperature combination (i.e. '90°C for 90 s') since it enables the FBOs to design a process that is compliant with the safety requirements while at the same time achieving a desired product quality. Establishing a risk based PrC however requires a full QMRA to be performed in order to inform the definition by risk managers of either an FSO or an ALOP.

Conflict of Interest

The authors declare no conflict of interest. The authors Winy Messens and José Cortiñas Abrahantes are employed by the European Food Safety Authority (EFSA). The present article is published under the sole responsibility of the authors and may not be considered as an EFSA scientific output. The positions and opinions presented in this article are those of the authors alone and do not necessarily represent the views or scientific works of EFSA.

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Table 1. Thermal inactivation of Hepatitis A virus (HAV) in bivalve molluscs samples

Sample	Inoculation strategy	Enumeration method (unit)	Initial concen-tration	Heating method	T (°C)	Times tested (min)	D-value (CI) (min)	z-value (°C)	Reference
Blue mussel homogenate (*Mytilus edulis*)	Inoculation of sample with HAV strain HM175	Plaque assay (PFU/mL)	6.73 log PFU/mL	Heating of homogenate (2 mL) in glass tubes in water bath[a]	56	0, 1, 2, 3	9.32 (3.26)[b]	12.97[b]	Bozkurt et al. (2014b)
					60	0, 0.33, 0.66, 1	3.25 (0.72)[b]		
					65	0, 0.25, 0.5, 0.75	2.16 (0.17)[b]		
					72	0, 0.16, 0.33, 0.5	1.07 (0.24)[b]		
Whole manila clams	Direct injection of HAV strain HM175 into the clam body	TCID ($TCID_{50}$)	5.4 log $TCID_{50}$/mL	Ten clams in bags were placed in gas-powered oven in steam mode[c]	60	0, 5, 10	5.00[d]	58.82[d]	Cappellozza et al. (2012)
					70	0, 5, 10	2.38[d]		
					80	0, 5, 10	2.29[d]		
Blue mussel homogenate (*Mytilus galloprovincialis*)	Inoculation of sample with HAV[j]	TCID ($TCID_{50}$)	5.5 log $TCID_{50}$/mL	Heating of homogenate (4 mL) in water bath[e]	60	0, 10, 15, 20, 25, 30	5.00[d]	33.11[d]	Croci et al. (1999)
					80	0, 3, 6, 10, 15	2.56[d]		
					100	0, 1, 2, 3, 5, 8	0.31[d]		
Green shell mussel homogenate	Inoculation of sample with HAV strain HM175	Plaque assay (PFU/g)	6 log PFU/g	Heating of mussel homogenate (10 g) in water bath[f]	60	0, 30, 60, 90, 120, 150[g]	109[d]	11.89[d]	Harlow et al. (2011)
					65	0, 30, 60, 90, 120, 150[g]	72[d]		
					70	10, 20, 30, 40, 50[g]	17[d]		
					75	0, 2, 4, 6, 8, 10[g]	7[d]		
Homogenate of dried	Inoculation of sample with	TCID ($TCID_{50}$)	7 log $TCID_{50}$/mL	Heating of homogenates	60	0, 5, 15, 30	7.93 (0.06)[d]	31.95[d]	Park and Ha

mussels	HAV strain HM175			(2 mL) in a 15 mL conical tube in a water bath[(h)]	85	0, 3, 6, 10	3.05 (0.13)[(d)]		(2015)
					100	0, 1	0.38 (0.38)[(d)]		
Digestive glands of soft-shell clams (*Mya arenaria*)	Inoculation of sample with HAV strain HM175	Plaque assay (PFU/mL)	5.47 log PFU/mL	Heating of a single digestive gland (1.5 to 2.5 g) of soft-shell clams in glass tubes in a water bath[(i)]	85	0, 1.5, 3, 5	1.72[(d)]	15.80[(d)]	Sow et al. (2011)
					90	0, 1.5, 3, 5	0.83[(d)]		

CI: Confidence Interval; HAV: Hepatitis A virus; PFU: plaque-forming unit; $TCID_{50}$: Tissue Culture Infective Dose.

[(a)]: The temperature was recorded using a control tube. The heat treatment time started when the desired temperature was reached in the sample.

[(b)]:The D-value (z-value) has been calculated by the authors.

[(c)]:The temperature was recorded by placing a probe in the bags.

[(d)]:The D-value (z-value) has been calculated by EFSA using the data as summarized in the paper.

[(e)]:The temperature of homogenate reached the desired temperature after a few seconds (s) (testing at 60 and 80°C) and reached 85°C after 30 s and 90°C after 1 min (testing 100°C).

[(f)]:The temperature was measured in the homogenate. The heat treatment time started when the desired temperature was reached in the sample.

[(g)]:The times tested have been provided by personal communication (Mrs Harlow, Health Canada, 17 July 2015).

[(h)]:The temperature recorded in aliquot of homogenate.

[i]:The temperature profile is provided in the paper. The heat treatment time started when the desired temperature was reached in the sample.

[j]:HAV isolated from the stool of a patient affected by acute hepatitis A.

Table 2. Thermal inactivation of surrogates for Norovirus (NoV), including Feline calicivirus (FCV) and murine NoV (MNV) in bivalve molluscs samples

Sample	Inoculation strategy	Enumer-ation method (unit)	Initial concen-tration	Heating method	T (°C)	Times tested (min)	D-value (CI) (min)	z-value (°C)	Reference
Blue mussel homogenate (*Mytilus edulis*)	Inoculation of sample with FCV-F9	Plaque assay (PFU/mL)	6.99 log PFU/mL	Heating of homogenate (2 mL) in glass tubes in water bath[(a)]	56	0, 1, 2, 3	3.33 (0.43)[(b)]	11.39[(b)]	Bozkurt et al. (2014a)
					60	0, 0.33, 0.66, 1	0.77 (0.20)[(b)]		
					65	0, 0.25, 0.5, 0.75	0.33 (0.03)[(b)]		
					72	0, 0.16, 0.33, 0.5	0.07 (0.01)[(b)]		
Blue mussel homogenate (*Mytilus edulis*)	Inoculation of sample with MNV-1	Plaque assay (PFU/mL)	6.74 log PFU/mL	Heating of homogenate (2 mL) in glass tubes in water bath [(a)]	56	0, 1, 2, 3	6.12 (0.81)[(b)]	11.62[(b)]	Bozkurt et al. (2014a)
					60	0, 0.33, 0.66, 1	2.64 (0.15)[(b)]		
					65	0, 0.25, 0.5, 0.75	0.41 (0.03)[(b)]		
					72	0, 0.16, 0.33, 0.5	0.18 (0.03)[(b)]		
Chopped digestive glands tissue of blue mussel (*Mytilus galloprovincialis*)	Inoculation of sample with FCV-F9 (ATCC VR-782)	TCID ($TCID_{50}$)	4.7 log $TCID_{50}$/mL	Heating of sample (3.6 g) in water bath	60	0, 3, 6, 10, 15	6.39[(c)]	29.76[(c)]	Croci et al. (2012)
					80	0, 3, 6, 10, 15	1.36[(c)]		
Abalone meat[(e)]	Inoculation of sample with MNV-1	Plaque assay (PFU/mL)	5.5 log PFU/mL	Heating of sample (5 g) in 50 mL conical tube in water	70	0, 1, 3, 5, 10	8.87	29.76[(c)]	Park et al. (2015)
					85	0, 1, 3, 5	2.78		

				bath					
Abalone viscera[(e)]	Inoculation of sample with MNV-1	Plaque assay (PFU/mL)	5.3 log PFU/mL	Heating of sample (5 g) in 50 mL conical tube in water bath	70	0, 1, 3, 5, 10	10.01	34.13[(c)]	Park et al. (2015)
					85	0, 1, 3, 5	3.64		
Digestive glands of soft-shell clams (*Mya arenaria*)	Inoculation of sample with MNV-1	Plaque assay (PFU/mL)	5.47 log PFU/mL	Heating of a single digestive gland (1.5 to 2.5 g) of soft-shell clams in glass tubes in a water bath[(d)]	85	0, 1.5, 3, 5	0.57[(c)]	-[(f)]	Sow et al. (2011)
					90	0, 1.5, 3, 5	0.56[(c)]		

CI: Confidence Interval; PFU: plaque-forming unit; $TCID_{50}$: Tissue Culture Infective Dose.

[(a)]: The temperature was recorded using a control tube. The heat treatment time started when the desired temperature was reached in the sample.

[(b)]: The D-value (z-value) has been calculated by the authors.

[(c)]:The D-value (z-value) has been calculated by EFSA using the data as summarized in the paper.

[(d)]:The temperature profile is provided in the paper. The heat treatment time started when the desired temperature was reached in the sample.

[(e)]: Abalone is a mollusc species but not a bivalve mollusc. It was included because of the scarcity of the data.

[(f)]: The z-value was not calculated as these data were not appropriate.

Table 3. Data used and structure of the Hepatitis A virus (HAV) risk assessment model to illustrate the risk-based approach by HAV for mussels consumption by adults in a EU Member State (MS)

Para-meter	Explanation	Distribution/Description	Data used	Reference
Pr_i	Initial HAV prevalence	$= 0.399$	Mussels in MS (n = 193)	Suffredini et al. (2017)
C_i	Initial HAV concentration (copies/g)	$\sim cumul(v;w)$ with $v = [(0,100,1000,5100)/10]$ [(a)] $w = [0, 0.617, 0.912, 1]$	Mussels in MS (n = 77)	Suffredini et al. (2017)
I	Inactivation during heat treatment (log copies/g)	Scenarios tested: $I = \{0,1,2,3,4,5\}$	-	-
S	Serving size for adult population (g)	$\sim cumul(v;w)$ with $v = [16.06, 16.06, 38.5, 161.98, 202.48, 202.48]$ $w = [0.05, 0.1, 0.5, 0.95, 0.975, 0.99]$	Mussels in MS (n = 112)	EFSA's food consumption database
C_c	HAV concentration at consumption time (log copies/g)[(b)]	$\sim \log(C_i) - I$	-	-
N_{cs}	HAV virions at consumption time (copies/serving)	$\sim Poisson(10^{C_c} \times S)$	-	-
Pr_c	HAV prevalence at consumption time	Prevalence after heat treatment[(c)]		
$P_{inf,cs}$	Probability of infection per contaminated serving	$= 1 - exp^{(-4\times10^6 \times N_{cs})}$	Exponential model for HAV dose-response	Bouwknegt et al. (2015)
$P_{inf,s}$	Probability of infection per	$\sim Pr_c \times P_{inf,cs}$	-	-

	serving			

[(a)]:Concentration divided by ten assuming that the digestive part sampled is 10% of the total mussel.

[(b)]:Only non-zero values were used.

[(c)]:Estimated based on Pr_i and the percentage of zero values in C_c.

Table 4. The estimated cumulative distribution of mussel servings with different concentrations of Hepatitis A virus (HAV) depending on the reduction of HAV achieved during heat treatment. Results are based on the QMRA model, parameters in Table 3[(a)] and initial concentration distribution (0 --log reduction column) representing an outbreak scenario.

HAV virions (copies/serving)	Reduction of HAV achieved during heat treatment (log copies/g)[(c)]					
	0[(b)]	1	2	3	4	5
< 1	60.4	59.9	61.3	67.6	86.0	97.4
< 10	60.5	60.4	69.1	92.6	99.9	100.0
< 100	61.0	67.6	92.8	100.0	100.0	100.0
< 1,000	68.5	91.9	100.0	100.0	100.0	100.0
< 10,000	92.4	100.0	100.0	100.0	100.0	100.0
< 100,000	100.0	100.0	100.0	100.0	100.0	100.0

[(a)]:Based on a Monte Carlo simulation with 10,000 iterations.

[(b)]:No thermal treatment applied.

[(c)]:Total reduction of HAV during heat treatment of mussels is from 0 to 5 logs. This is an illustration of the risk-based approach.

Table 5. Effect of the tested scenarios for the total reduction of Hepatitis A virus (HAV) during heat treatment (from 0 to 5 logs) of mussels on the probability of HAV infection per serving

Probability of HAV infection per serving	Total HAV reduction during heat treatment (log copies/g)[(a)]					
	0	1	2	3	4	5
Average	1.08E-02	1.15E-03	1.34E-04	1.15E-05	1.12E-06	1.21E-07
Median	4.59E-03	4.88E-04	6.69E-05	5.17E-06	5.56E-07	1.08E-07
95th	4.50E-02	4.77E-03	5.27E-04	4.52E-05	3.34E-06	2.16E-07
99th	8.91E-02	1.02E-02	1.01E-03	8.40E-05	5.00E-06	3.24E-07

[(a)]: This is an illustration of the risk-based approach.

Table 6. F_{90} values for different Performance Criteria of Hepatitis A virus (HAV) inactivation during bivalve molluscs heat processes calculated based on the HAV thermal inactivation model

Performance Criterion (PC)	Process Criterion (PrC): F_{90} (s)		
Total HAV reduction (log PFU/g)	Mean	Lower 95% CI	Upper 95% CI
1	54	36	78
2	108	72	156
3	162	108	234
4	216	144	312
5	270	180	390
6	324	216	468

CI: Confidence Interval; PFU: plaque-forming units.

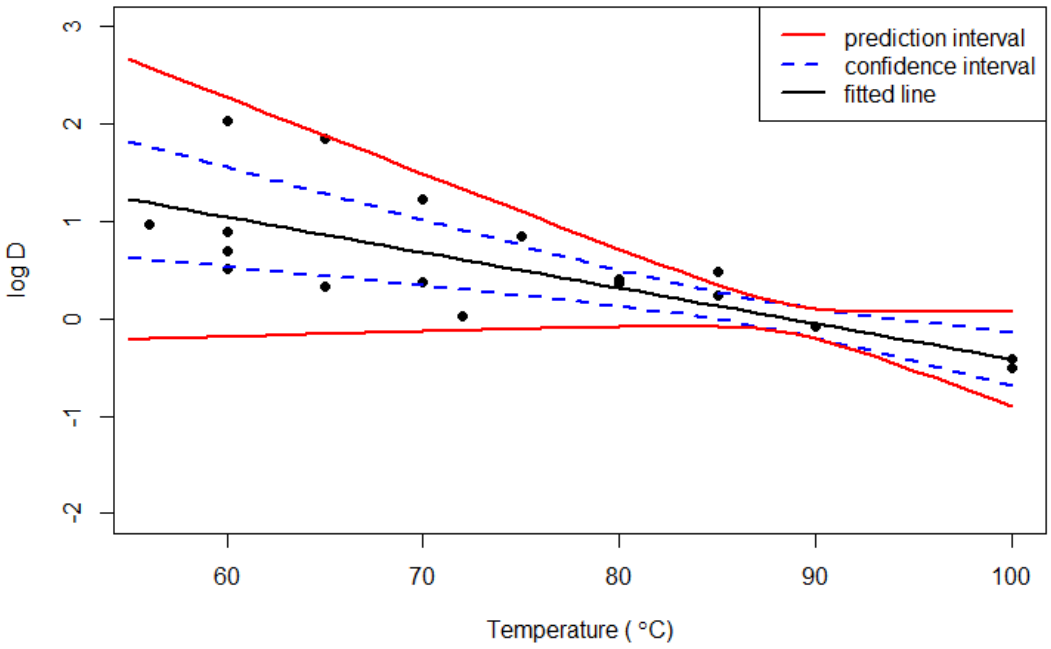


Figure 1. Linear regression of log D versus temperature for Hepatitis A virus (HAV) inactivation in mollusc matrices. The 95% Prediction Interval for prediction of observations of log D and the Confidence Interval of mean log D for the parameters are also shown.

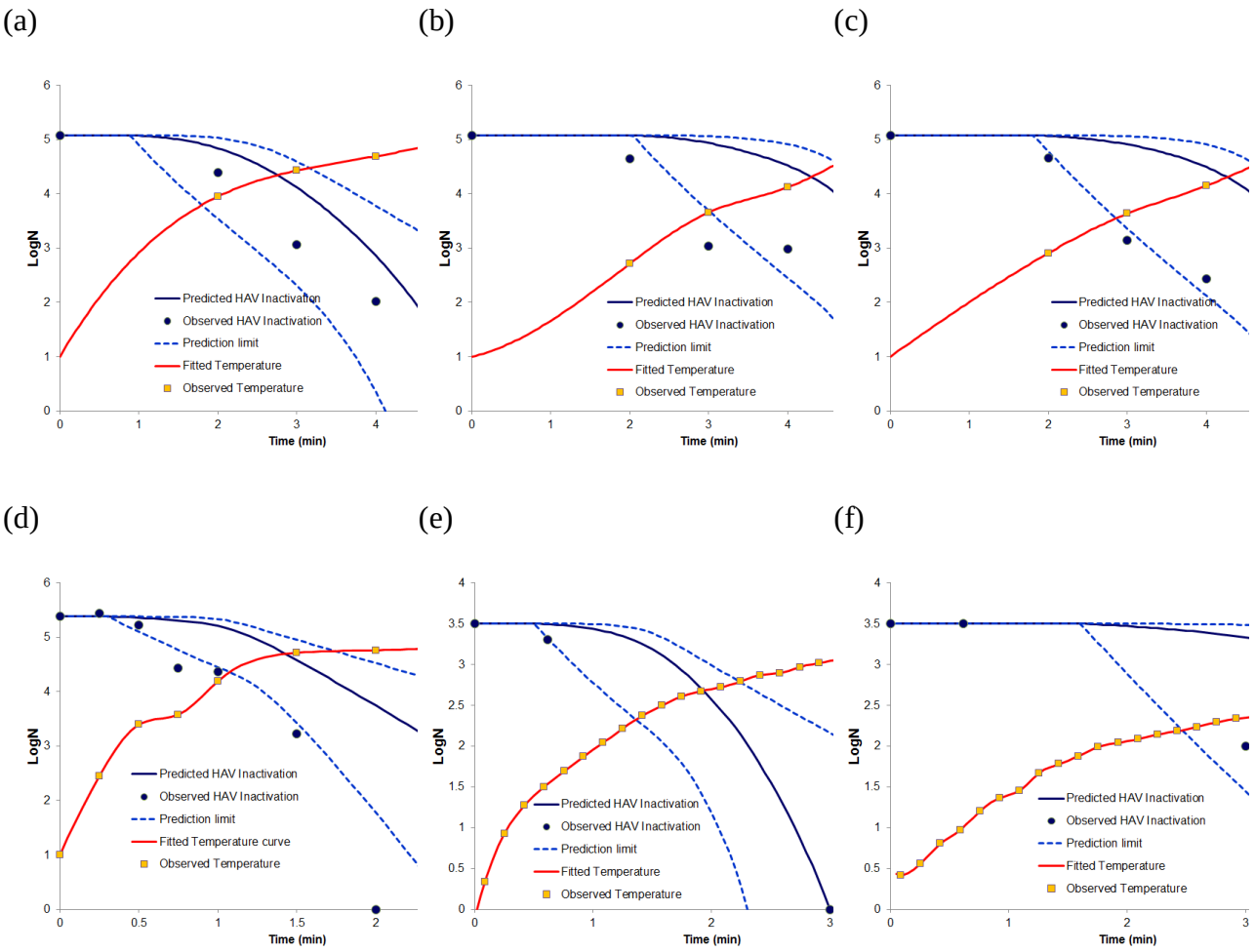


Figure 2. Comparison between observed Hepatitis A virus (HAV) inactivation in mussels and predicted inactivation by the HAV thermal inactivation model. (a) Harlow et al. (2011), bottom layer; (b) Harlow et al. (2011), middle layer; (c) Harlow et al. (2011), top layer; (d) Millard et al. (1987); (e) Hewitt and Greening (2006), boil; (f) Hewitt and Greening (2006), steam.

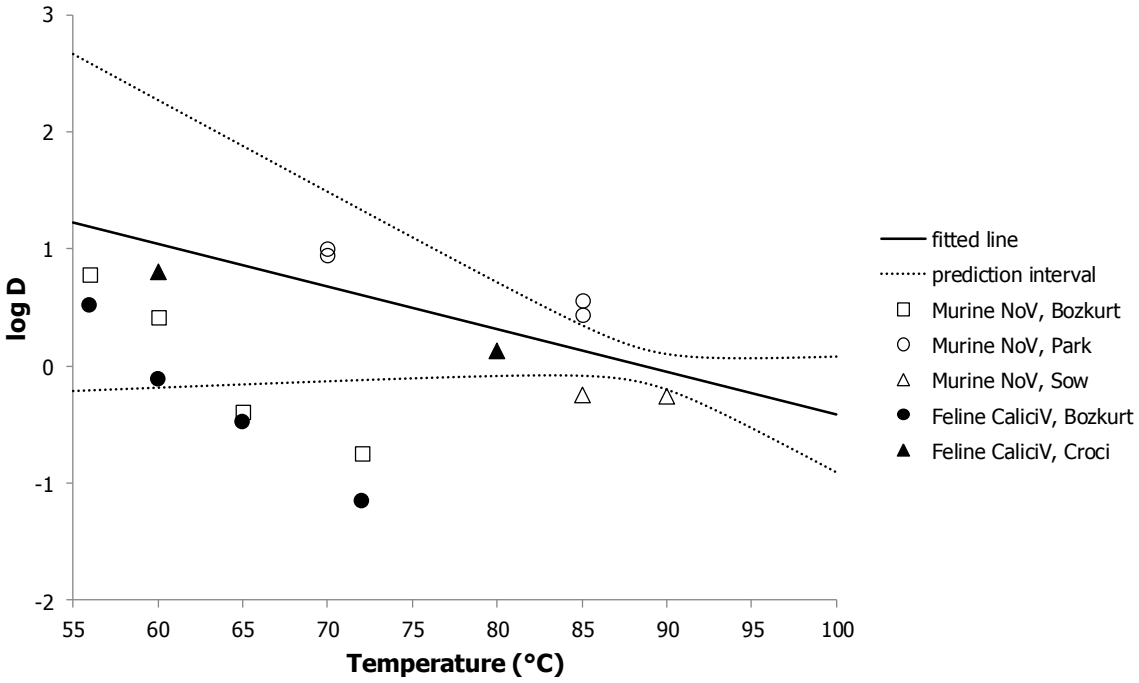
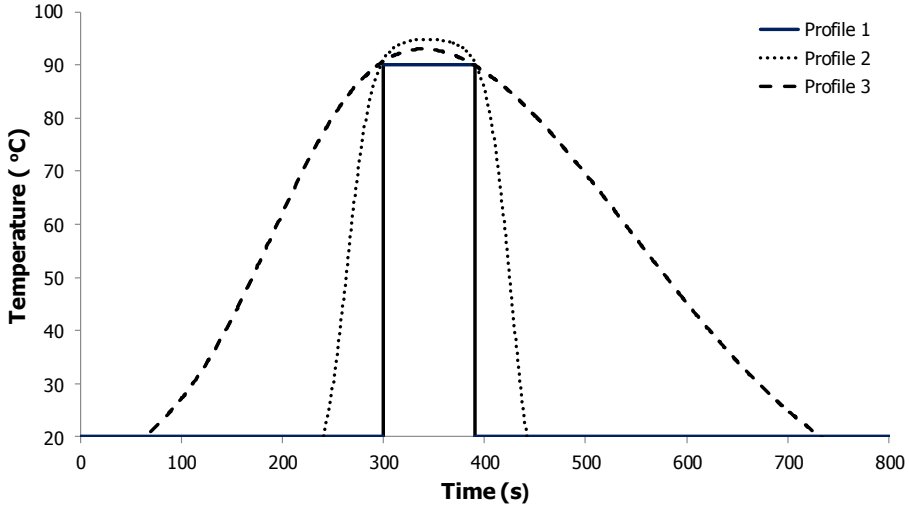


Figure 3. Log D values reported for different viruses in various mollusc matrices at different temperatures compared to the linear relationship developed based on Hepatitis A virus (HAV) data (fitted line and 95% prediction interval)

(a)



(b)

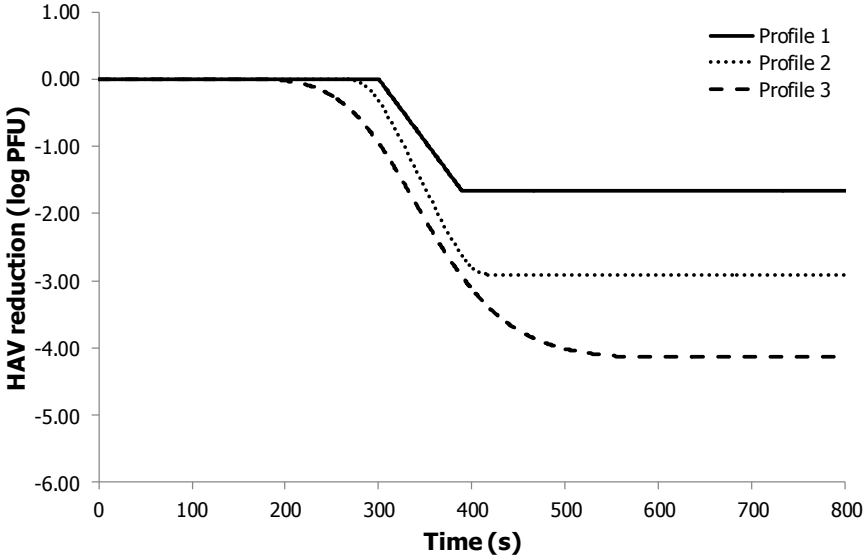


Figure 4. Examples of different heat processing time--temperature profiles complying with the '90°C for 90 s' requirement (a) and their predicted Hepatitis A virus (HAV) inactivation (b)

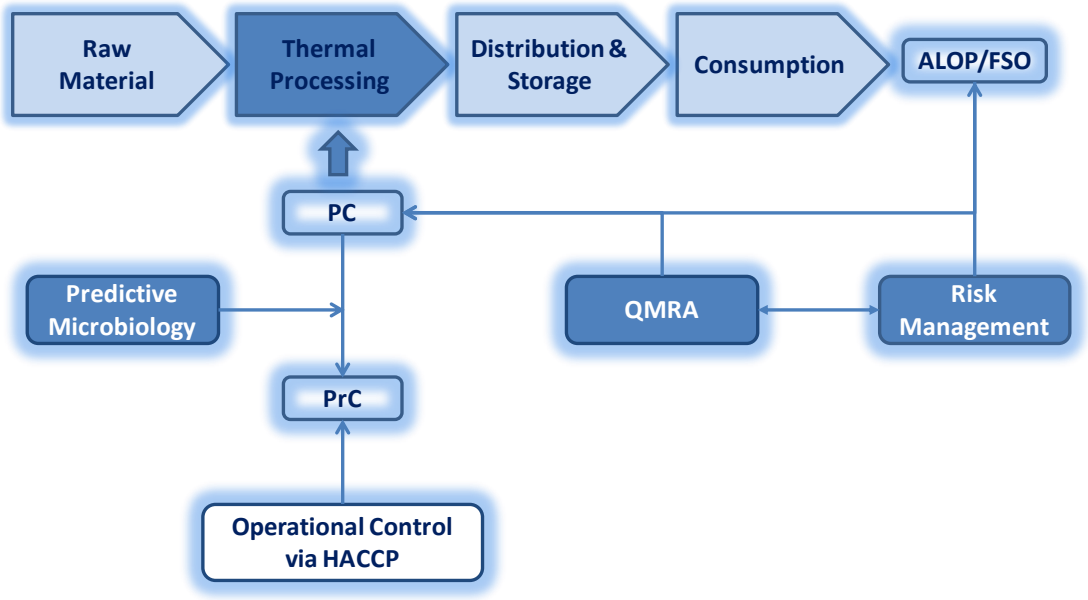


Figure 5. Structure of a risk-based design for thermal processing of foods

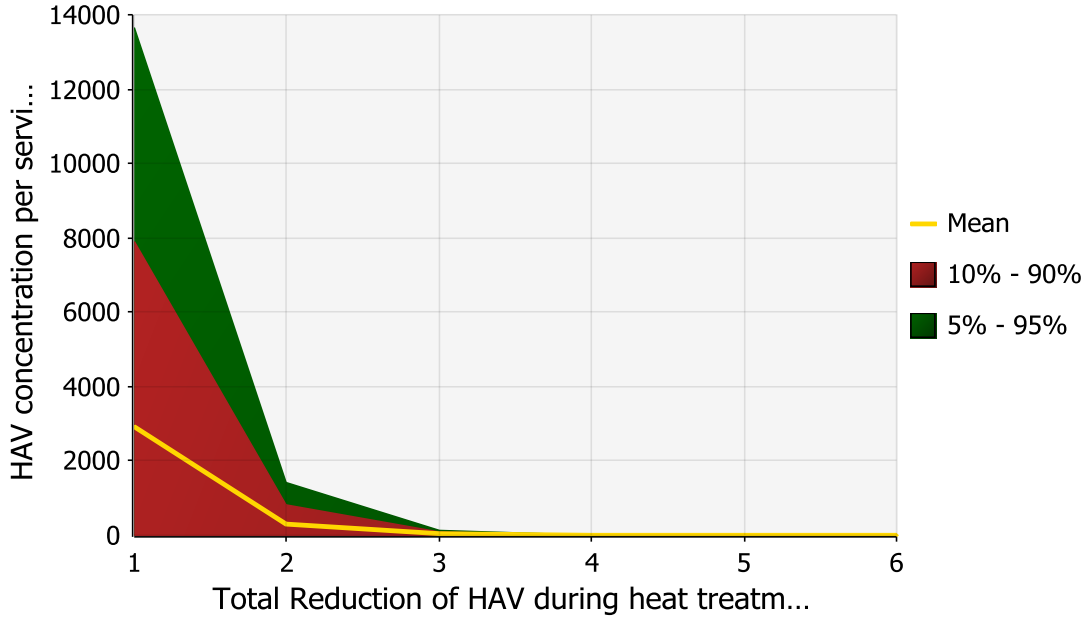


Figure 6. Summary trend graph on the effect of total reduction of Hepatitis A virus (HAV) (log units) during heat treatment of mussels on the HAV concentration per serving assuming an initial concentration from an outbreak scenario

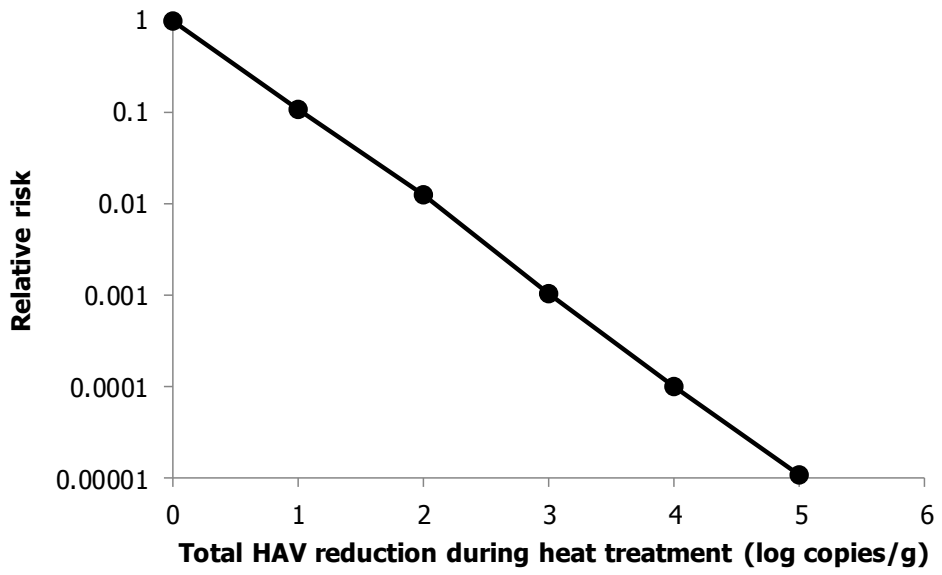


Figure 7. Effect of the total reduction of Hepatitis A virus (HAV) during heat treatment on the relative risk of HAV infection

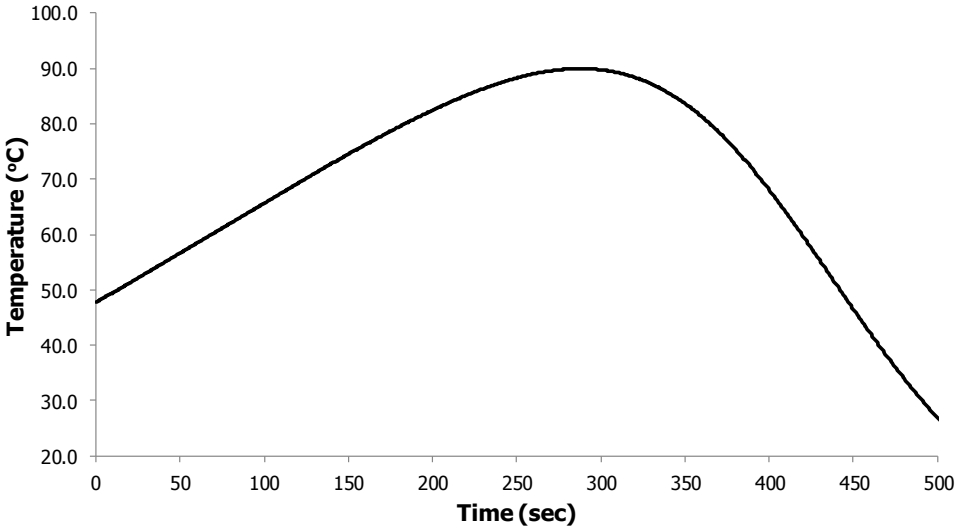


Figure 8. Example of a heat processing time--temperature profile with F_{90} = 179 s