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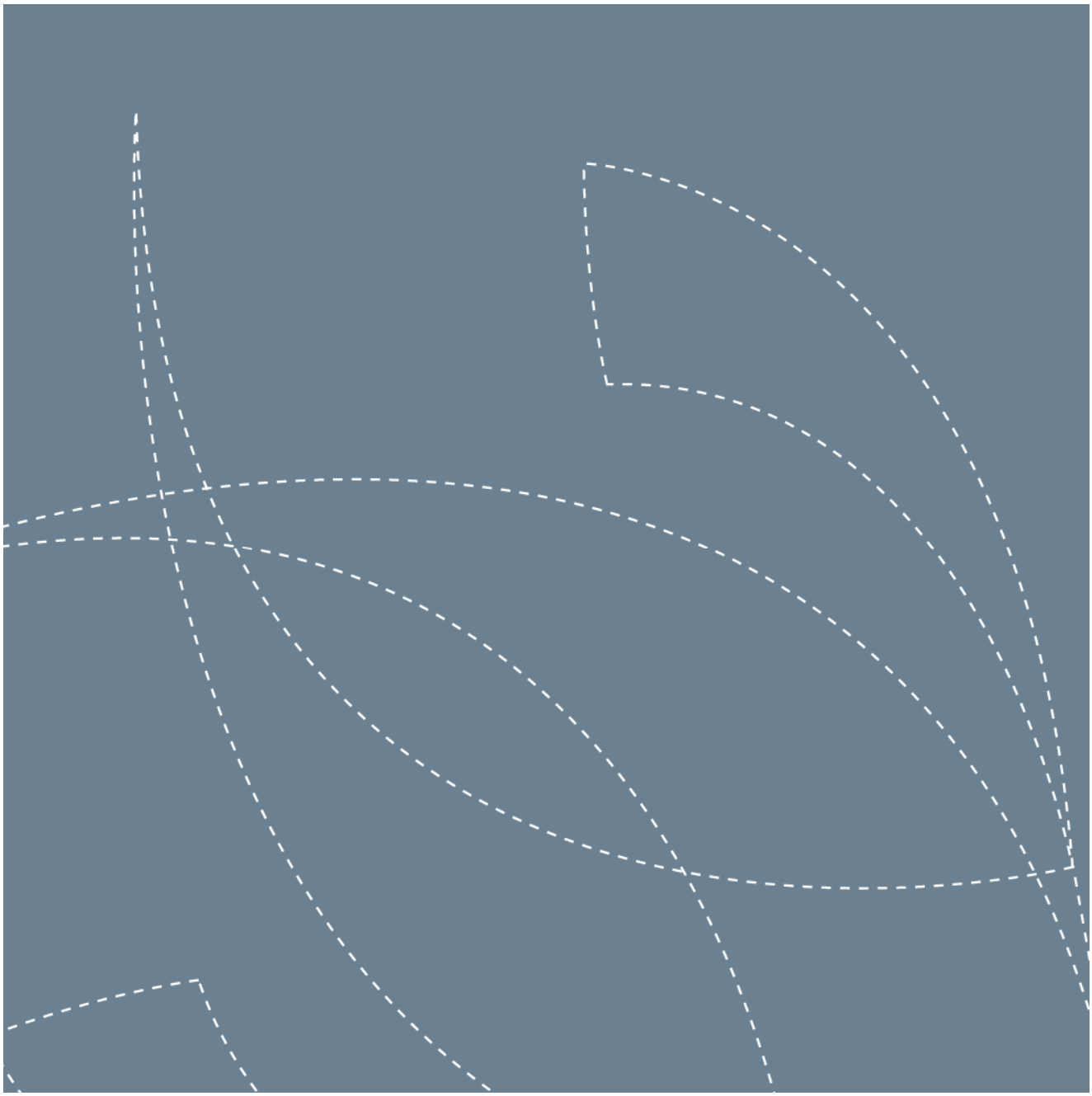
Inactivation of pathogenic microorganisms in fish by-products

Sub-project IPN-virus

Halvor Nygaard, Nofima and Mette Myrmel, Veterinærinstituttet



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Main office in Tromsø
Muninbakken 9–13
P.O. box 6122
NO-9291 Tromsø
Norway
Tel.: +47 77 62 90 00
Fax: +47 77 62 91 00
E-mail: nofima@nofima.no

Internet: www.nofima.no

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Nofima Marin AS
Nofima Ingrediens
Kjerreidviken 16
NO-5141 Fyllingsdalen
Norway
Tel.: +47 55 50 12 00
Fax: +47 55 50 12 99
E-mail: ingrediens@nofima.no

Internet: www.nofima.no

Nofima Marin AS, Nofima Ingrediens
Kjerreidviken 16, NO-5141 Fyllingsdalen, Norway
Tel.: +47 55 50 12 00, Fax: +47 55 50 12 99
ingrediens@nofima.no
www.nofima.no

Business reg. no.: NO 964 441 898 VAT

Veterinærinstituttet
Ullevålsveien 68, NO-0106 Oslo
Tel.: +47 23 21 60 00, Fax.: +47 23 21 60 01
postmottak@vetinst.no
www.vetinst.no

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Author(s):
Halvor Nygaard, Nofima and Mette Myrmel, Veterinærinstituttet

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Summary:

The aim of the present study was to determine the inactivation effect on IPNV resulting from heat treatment of fish by-products at different temperatures. Furthermore, the inactivation effect of a new alternative processing method intended for category 2 fish material was examined. The method involves size reduction, organic acid treatment and heat treatment (85 ºC for 25 minutes).

Decimal reduction times (D-values) and temperature coefficients (z-values) were determined after heat treatment of IPNV in artificial media resembling fish silage with regard to its content of water soluble protein and salt. The inactivation curves obtained in this study were bi-phasic. A rapid initial loss of infectivity (approximately 0,7 Log_{10} reduction) was followed by a much slower exponential decrease. D-values for thermal inactivation at 60, 70, 80 and 90 ºC ranged from 290 to 0,5 minutes. The temperature increase (z-value) needed to obtain a ten-fold reduction of D-values was approximately 9,8 ºC. Surprisingly, the experiments showed that IPNV infectivity was better maintained at pH 4 (formic acid) than at pH 7. However, the heat resistance of IPNV was little influenced by pH.

Based on the D- and z-values obtained during heat exposure of IPNV in artificial media, IPNV inactivation during the heating phase of the new processing method (85 ºC, 25 minutes) was calculated to 27 Log_{10} reductions. Even if IPNV is virtually unaffected by exposure to formic acid (pH 4), the heating phase alone will be fully sufficient to ensure adequate inactivation of IPNV during the complete process.

In inactivation studies using fish suspension as a matrix, no infective IPNV could be recovered from the suspension at any step. The results indicate that the fish suspension reduces the IPNV infectivity. Extended studies on other batches of fish suspensions should be performed before a general conclusion on the effect of fish suspensions can be made.

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1 Introduction

The project was initiated by Norwegian Seafood Federation (FHL) and funded by Rubin, a foundation working for increased and more profitable utilization of by-products from the fisheries and fish farming in Norway.

The aim of the present investigation was to determine the inactivation effect on IPN virus resulting from heat treatment of fish by-products at different temperatures. Furthermore, the effect of a new alternative processing method, intended for category 2 fish material, was examined. The processing method involves size reduction, organic acid treatment and heat treatment (85 ºC for 25 minutes).

Category 2 material of fish origin is mainly derived from dead fish collected at aquaculture farms on a daily basis as part of good hygiene practice. The mortality could be due to infectious or non-infectious diseases. Another major source is dead fish from disease outbreaks, whether the cause is listed or non-listed diseases. This may include fish without clinical signs of disease, killed in order to eradicate epizootic diseases. Category 2 material can also contain dead fish collected after mass mortality caused by e.g. sudden changes in water quality or jellyfish invasions. Less common is fish containing residues of veterinary drugs exceeding permitted levels.

Industries processing fish by-products shall ensure that pathogens in raw materials are adequately inactivated during the process. IPN virus is commonly regarded as the most heat resistant among fish-pathogenic microorganisms. However, few exact data on its temperature-dependant inactivation are available. Such data are therefore urgently needed by the processing industry as well as by supervisory and regulatory authorities.

According to the Commission Regulation (EC) 1774/2002, even for category 2 material of fish origin, high pressure sterilization is required before transformation into biogas or organic fertilizers. The regulation allows for the adaptation of an alternative method for the processing of such material, but so far none such methods are approved. The requirement for high pressure sterilization also represents an unnecessary use of energy and emission of CO_2, and could be considered unsustainable. FHL has applied for approval of the new alternative processing method for category 2 material of fish origin, according to Regulation (EC) 1774/2002. The new results and documentation obtained in the present project will support the application.

2 Materials and methods

2.1 Preparation of gamma-irradiated fish suspension

By-products from slaughtering of Atlantic salmon and sea trout were provided by Biomega AS. Head/backbones were coarsely minced and subsequently mixed with equal parts of viscera before new mincing and homogenisation using an Ultra-Turrax knife homogenizer. Finally, the suspension was passed through a metal sieve ASTM 8 (mesh 2.36 mm opening), distributed in 500 ml capacity screw capped polyethylene bottles and frozen at ≤ -20 °C.

The material was sent in a frozen condition to Institute for Energy Technology, N-2027 Kjeller, Norway, and exposed to 10.3 kGy of gamma irradiation, in order to eradicate its indigenous microbial flora. After irradiation, the fish suspension was stored frozen until use. According to The Codex General Standard for Irradiated Foods (CAC/RS 106-1979) the overall average dose absorbed by a food subjected to radiation processing can be up to 10 kGy. The treatment is considered to affect nutritious properties less than thermal processing and it should neither affect other properties of relevance to our experiments.

2.2 Preparation of artificial media

Data on the chemical composition of typical fish silage was provided by Hordafor AS. The content of water soluble protein and salt in the water phase of the silage was calculated (Table 1).

Table 1 Typical composition of raw complete fish silage and calculated content of water-solube protein and salt in its water phase

Parameter	Unit	Fish silage complete (typical)	Fish silage water phase (calculated)
Fat	%	24,5	
Dry matter	%	36,5	
Fat-free dry matter	%	12,0	
Protein, total	%	9,4	
Protein, water-soluble	%	8,0	10,6
Salt	%	0,3	0,4

For the preparation of artificial media (Table 2), Bacteriological peptone[1] (Oxoid L 37) was chosen due to its high protein (87,5 %) and low salt content (1,6 %). The content of water soluble protein and salt in the finished media should be similar to that found in typical fish silages. The pH was adjusted to be 7,0 or 4,0 after sterilization. The media were sterilized in an autoclave at 121 °C for 15 minutes.

[1] A mixed pancreatic and papaic digest of animal proteins

Table 2 Preparation of artificial media with neutral and low pH

Ingredient	Unit	Neutralized medium (1N NaOH)	Acidified medium (80 % Formic acid)
Bacteriological peptone	g	121,0	121,0
NaCl	g	2,0	2,0
Water	ml	1000,0	1000,0
pH		Adjusted to 7,0 by NaOH	Adjusted to 4,0 by formic acid

2.3 Preparation of salmon oil sample

A fresh sample of salmon oil was provided by Nofima BioLab (Sample ref. no.: 2010-00138). The oil sample was centrifuged at 8000 x G for 30 minutes in order to remove impurities. The results of chemical and bacteriological analyses of the prepared salmon oil are shown in Table 3.

Table 3 Composition of salmon oil sample

Parameter	Unit	Result
Free fatty acids	%	2,6
Water	%	0,24
Impurities	%	< 0,01
Anisidin number		14,0
Peroxide number	meq peroxide/kg oil	6,4
Enterobacteriaceae	per g	< 10
Salmonella	in 25 g	not detected

2.4 Preparation of IPNV suspension

IPNV (serotype Sp) was grown on BF-2 cells in 75 cm^2 flasks at 15 °C using EMEM with 10% FBS, 4mM L-glutamin and 50µg/ml Gentamicin. Three rounds of virus propagation were performed, giving virus titers of 6-7 Log_{10} $TCID_{50}$/ml.

2.5 Examination of IPNV quantification methods

RT-PCRs and cell cultures were examined for their feasibility in quantification of infective IPNV in fish suspension. RT-PCR detects viral RNA and may give positive results on infective and non infective virus particles as long as the RNA is partly intact. The processing method (heat) should therefore degrade viral RNA if RT-PCR is to be used for quantification. Cells may be sensitive to toxic substances in the fish suspension.

RT-PCR, real time and traditional

To examine the effect of heat on the integrity of IPNV RNA, aliquots of the virus suspension were incubated at 85 °C for 25 minutes, 5 hours and 24 hours.

RNA was extracted with the EasyMag extraction robot and used as template in a real time TaqMan RT-PCR and two traditional RT-PCRs.

Cell culture

Two cell lines (BF-2 and CHSE) were tested for their sensitivity to substances in the fish suspension. The suspension was centrifuged at 2500 rpm for 15 min to remove particulate material. Tenfold dilutions of the supernatant (with and without neutralization) were added to the cells, in duplicates. To increase the visibility of the cells, a thin floating layer of fat had sometimes to be removed from the lowest dilutions of the fish suspension.

2.6 IPNV distribution in oil/water mixture

To estimate the concentration of IPNV in the removed fat fraction, the distribution of IPNV in an oil/water mixture was examined. IPNV was added to a 1:1 mixture of water and pure fish oil, giving a 1:10 dilution of the virus suspension. The mixture was shaken vigorously and left for sedimentation at 4 °C for 1 h. Samples were taken in triplicate from the fat and water fractions. RNA was extracted and IPNV was quantified by real time PCR, using tenfold dilutions of the virus suspension as a standard.

2.7 Quantification of infective IPNV

Infectious IPNV was quantified by titration in BF-2 cells (without FBS) in 96 well plates with 6 parallels of each dilution. The cells were screened for cytopathogenic effect after 7 days. Viral titer was determined by the method of Kärber (1979).

2.8 Chemical and bacteriological analyses

Chemical and bacteriological analyses were performed by Nofima BioLab, according to the methods referred in Table 4.

Table 4 Method references

Parameter	Method reference
Salt	AOAC 937.09
Raw protein (Kjeldahl)	ISO 5983
Raw protein, water-soluble	Internal
Dry matter	ISO 6496
Ash	ISO 5984
Fat (Ethylacetate extraction)	NS 9402
Free fatty acids	AOCS Ca 5A-40
Water (Carl Fisher)	AOCS Ca 2e-84
Impurities in oil	ISO 663
Anisidin number	AOCS Cd 18-90
Peroxide number	AOCS Cd 8b-90
Aerobic microorganisms	Nordval 012
Enterobacteriaceae	ISO 21528-2
Anaerobic sulphite-reducing bacteria	NMKL 56
Salmonella (PCR)	Nordval 023

2.9 Heat treatment

The IPNV suspension was diluted 1:10 in the heating media (fish suspension or artificial medium). Portions of inoculated medium, 2,2 ml in 14 x 100 mm glass reagent tubes, were submerged in water baths set to different temperatures. The temperature of the water baths was monitored during the experiments, using an accredited calibrated logger system (EBRO EBI-125). Tubes were constantly stirred during the first 2 minutes of heat treatment in order to facilitate heat transfer. After heat treatment for predetermined periods of time, the tubes were transferred to an ice/water mixture for rapid cooling. The glass tubes were intermediately kept refrigerated before the samples were transferred to sterile stopped plastic tubes (Greiner, cat. no. Z617792-1000EA). The samples were sent chilled in insulated containers and delivered at The National Veterinary Institute 24-48 hours after heat treatment. The course of temperature change in the media during heating or cooling was determined prior to the inactivation experiments, using an accredited calibrated digital thermometer (Anritsu HA-250K).

2.10 Modelling of new processing method

Pre-incubation

The IPNV suspension was diluted 1:10 in fish suspension and the inoculated fish suspension (22,0 gram) was incubated at 1,2 ± 0,2 °C for 24 hours.

Acid treatment

Inoculated and pre-incubated fish suspension were adjusted to pH 4,0 by adding 80 % formic acid. The acidified suspensions were stored at 20 ± 1 °C for 24 hours.

Heat treatment

Portions of the acid treated suspension, 2,2 ml in 14x100 mm glass reagent tubes, were submerged in a water bath set to 85,0 °C. Temperature monitoring of the water baths, heat treatment, cooling and shipment were done according to the procedure outlined in Chapter 2.9.

2.11 Calculation of D- and z-values

Thermal inactivation of micro-organisms, at constant temperature, is ideally an exponential (first-order) function. When the logarithmic number of survivors is plotted versus time, the resulting inactivation curve is a straight line and the negative reciprocal of its slope is the D-value. In practice, the points obtained seldom fit perfectly into a straight line and the D-value is therefore calculated from the slope of its regression exponential curve.

Similarly, the relationship between Log D-value and the corresponding exposure temperatures is also an exponential function and the negative reciprocal of its slope is the z-value (often referred to as the temperature coefficient).

The relationship between D-values and temperature is expressed by Bigelow's equation (Bigelow, 1921):

Log Dx = (Ty – Tx)/z + Log Dy

where Dx and Dy are decimal reduction times at temperature Tx and Ty, and z is the temperature coefficient. With Bigelow's equation, D-values can be estimated for other temperatures than those experimentally examined.

Definitions:

The D-value (Decimal reduction time) is the time needed at a certain temperature to kill 90 % of the organisms being studied.

The Z-value (Temperature coefficient) is the temperature change needed to change the decimal reduction time by a factor of 10.

3 Results

3.1 Characterization of fish suspension

The fish suspension had a high content of fat and a relatively low content of protein and ash (table 5). The main reason is probably that sieving of the homogenized material removed more bone fragments than other constituents. In industrial scale silages, bone sedimentation is frequently encountered. The composition of the fish suspension is therefore probably not very different from industrial silages after bone sedimentation. In spite of a high content of dry matter, the suspension had a low viscosity, allowing effective blending with IPN virus or acid and rapid temperature equilibration during heating and cooling.

By-products from farmed fish may contain a microbial flora able to interfere with quantitative analyses of added model organisms. The suspension was intended for use in experiments with Salmonella and selected fish pathogenic bacteria in addition to the IPNV experiments. The suspension was therefore exposed to a low dose of gamma irradiation in order to minimize its inherent microbial flora while still retaining other properties of relevance to our experiments.

Jamdar and Harikumar (2007) demonstrated that 5 and 10 kGy reduced the number of viable bacteria in poultry viscera by 4 and 6 Log_{10} cycles, respectively, while 20 kGy resulted in sterility. Even 20 kGy had little effect on other examined organoleptic and biochemical parameters. Hwang and Hau (1995) showed that 10 kGy had little effect on the activity of proteolytic enzymes and neither resulted in any structural changes of myofibrils. Similar doses are known to be effective against viruses (De Benedictis et al. 2007). The irradiation dose used by us (10 kGy) is the maximum dose allowed in foods, according to The Codex General Standard for Irradiated Foods (CAC/RS 106-1979).

Table 5 Analyses in fish suspension before and after gamma irradiation (10,3 kGy)

		Results	
Analysis	Unit	Before irradiation	After irradiation
Raw protein Kjeldahl N*6.25	%		6,6
Fat (Ethylacetate extraction)	%		54,4
Total dry matter	%		60,2
Ash	%		0,8
Aerobic microorganisms	Number per. g	1.000.000	< 250
Enterobacteriaceae	Number per. g	1.500	< 10
Anaerobic sulphite-red. bacteria	Number per. g	890	< 10
Salmonella	Detected/Not det. in 25 g	Not detected	Not detected

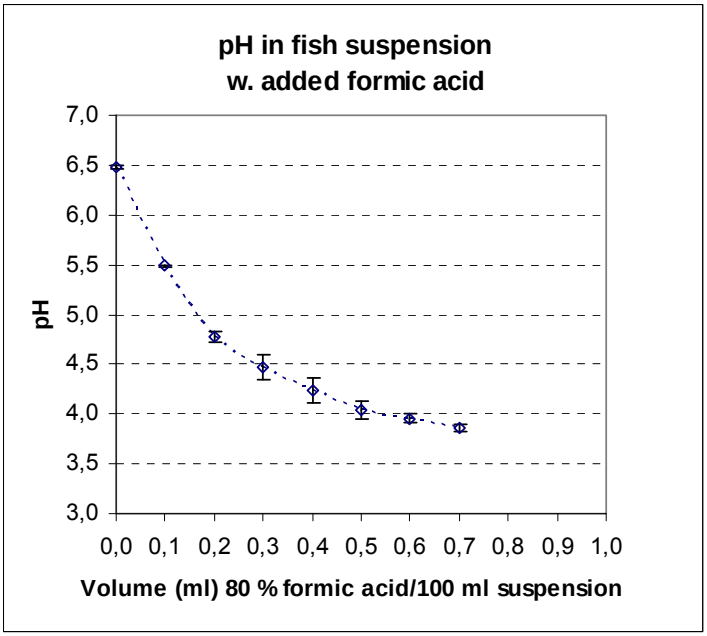


Figure 1 pH in fish suspension versus amount of added 80 % formic acid. The points represent the average and standard deviation for three parallel experiments.

In a separate examination (Figure 1) it was demonstrated that the addition of 0.6 ml 80 % formic acid to 100 ml fish suspension resulted in pH 4.0. This concentration of 0.6 % is low compared to what is presently used in the fish silage industry (1.0-3.5 %, depending on the composition of the fish material) (RUBIN, 1993). The low consumption of acid is probably due to a low content of bones and a high content of fat.

3.2 Characterization of artificial medium

Table 6 Analyses in artificial medium

Analysis	Unit	Result
Raw protein (Kjeldahl)	%	10,2
Fat (Ethylacetate extraction)	%	0,7
Total dry matter	%	10,9
Salt	%	0,3

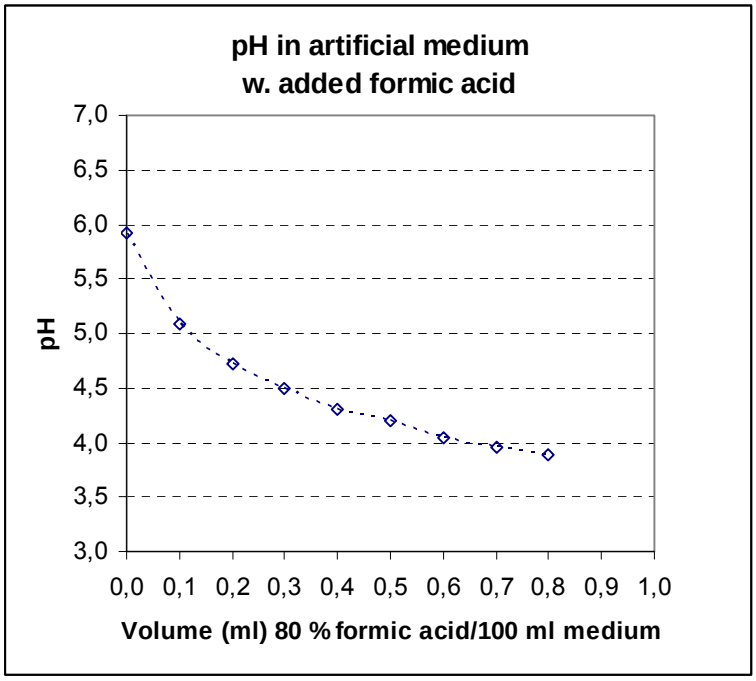


Figure 2 *pH in artificial medium versus amount of added 80 % formic acid.*

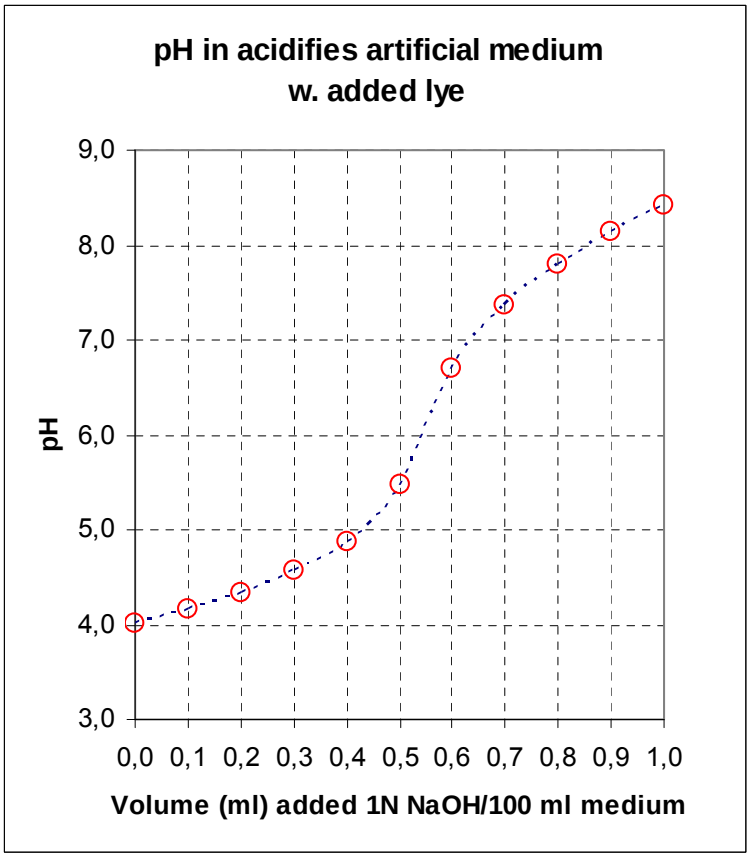
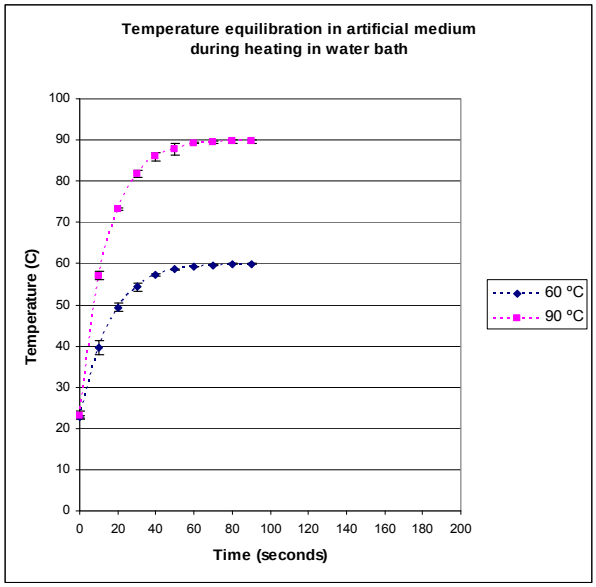


Figure 3 *pH in acidified artificial medium versus amount of added 1N NaOH.*

3.3 Temperature profiles during heating and cooling

a)



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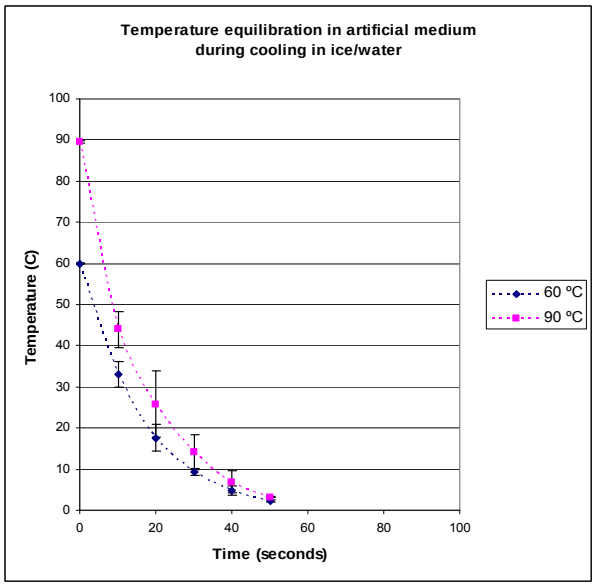
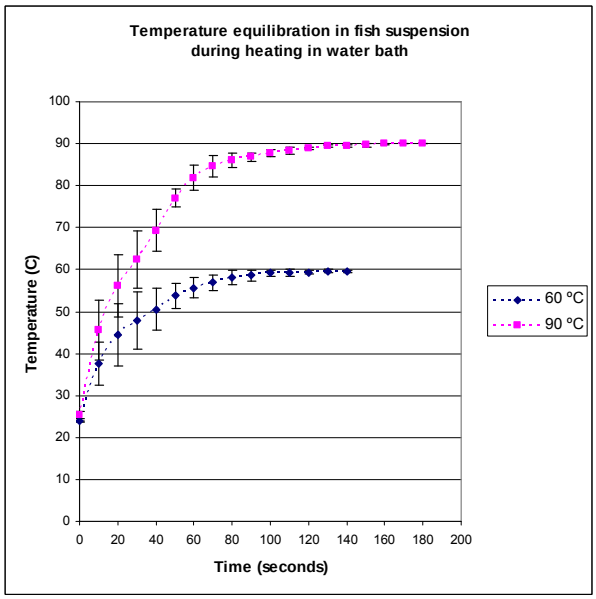


Figure 4 Temperature equilibration in artificial medium (2,2 ml in 14x120 mm glass tubes) during heating in water bath (a) or cooling in ice/water (b). Each data point is the average of 3 parallel tests. Vertical bars represent the standard deviation.

a)



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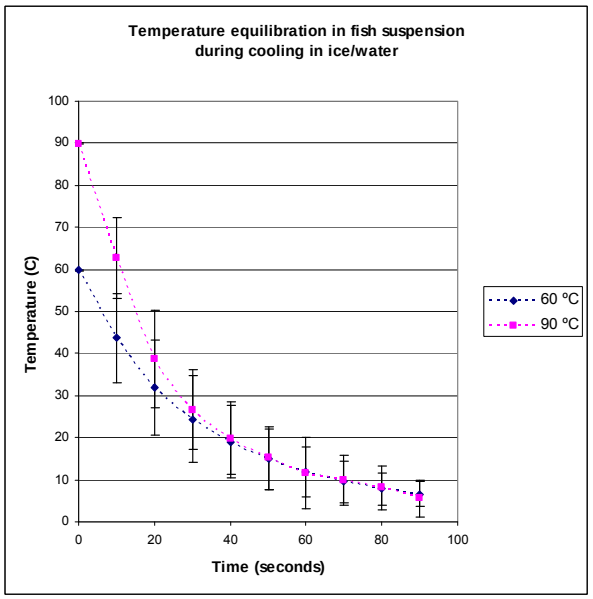


Figure 5 Temperature equilibration in fish suspension (2,2 ml in 14x120 mm glass tubes) during heating in water bath (a) or cooling in ice/water (b). Each data point is the average of 3 parallel tests. Vertical bars represent the standard deviation.

3.4 IPNV quantification methods

Heat treatment of IPNV at 85 ºC for 25 min did not degrade the viral RNA. After 5 h and 24 h a 2-3 Log and 4-5 Log reduction in RNA copies was found by real time PCR, respectively. By traditional PCR, positive samples were found after 25 min and 5 h.

The BF-2 cells showed higher resistance than the CHSE cells to the components in the fish suspension. At a 1:100 dilution both the neutralized and original suspension had no detrimental effect on the BF-2 cells.

PCR was rejected for the quantification of IPNV and the BF-2 cells were chosen for quantification of infective viruses.

3.5 IPNV distribution in oil/water mixture

The IPNV concentration was more than 1 Log higher in water compared to the oil fraction. The oily layer could therefore be removed from wells without substantially influencing the amount of virus.

3.6 IPNV inactivation

3.6.1 Pre-investigation

Artificial media inoculated 10:1 with IPNV suspension (6,6 Log_{10} $TCID_{50}$/ml) was heat treated at 70 and 85 ^{o}C for 25 minutes, in order to determine appropriate combinations of exposure times and temperatures for the subsequent inactivation experiments. Based on the results (Table 7) it was decided to use temperatures in the range between 60 and 90 ^{o}C for heat treatment at both pH 7,0 and 4,0.

The results of the pre-investigation indicated that IPNV may be more stable during cold storage in artificial medium at pH 4,0 than at pH 7,0.

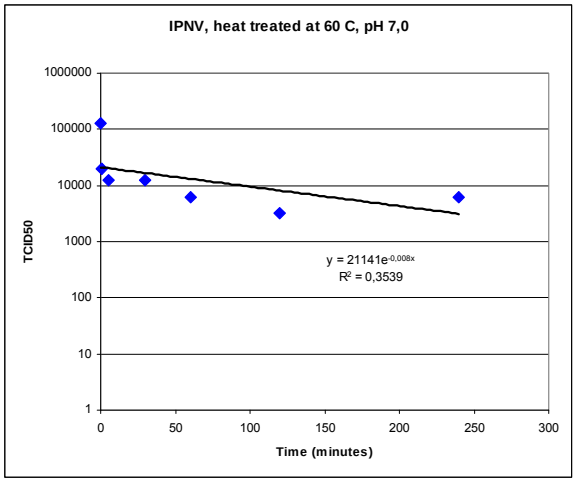
Table 7 Log_{10} IPNV titer ($TCID_{50}$/ml) after heat treatment in pH 7,0 and pH 4,0 (formic acid) artificial media. The detection limit of the assay is 2,8 Log_{10} $TCID_{50}$/ml.

	Heat treatment		
	No heating	70 ºC/25 minutes	85 ºC/25 minutes
pH 4,0	6,1	5,1	<2,8
pH 7,0	4,5	3,8	<2,8

3.6.2 Heat inactivation in artificial medium at pH 7,0

Artificial medium at pH 7,0 were inoculated 10:1 with an IPNV suspension containing approximately 6,3 Log_{10} $TCID_{50}$/ml before heat exposure.

a)



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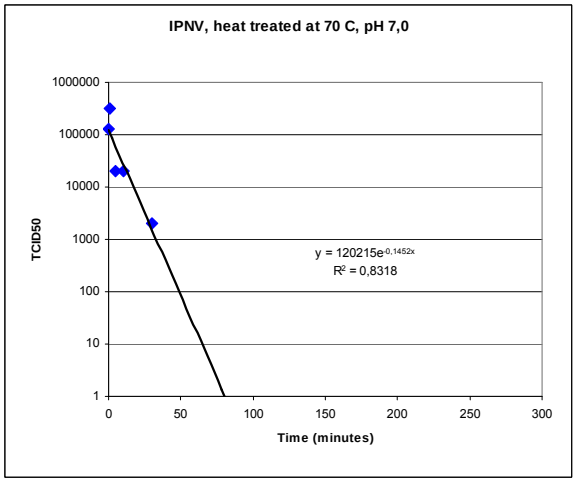

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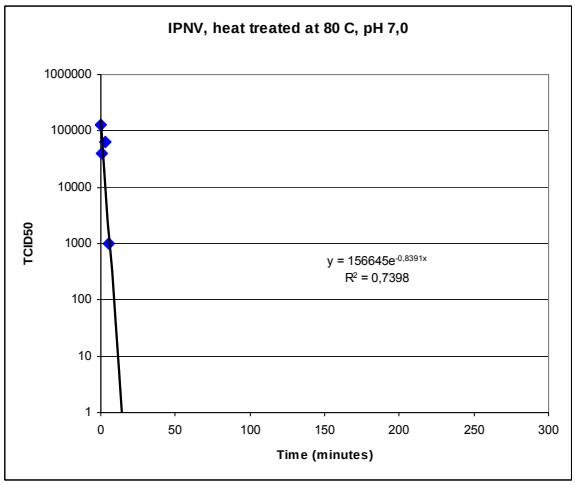

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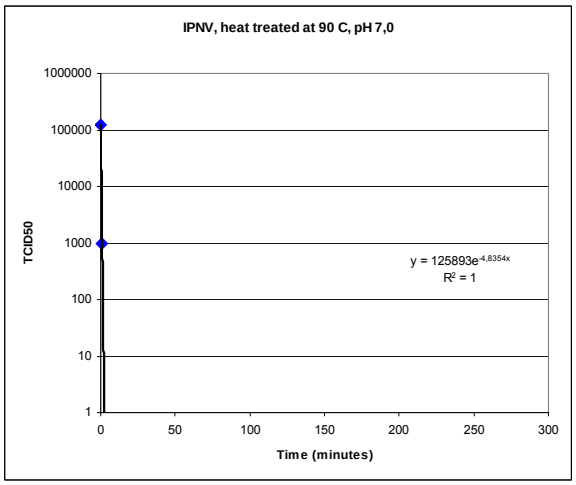


Figure 6 Inactivation curves for IPNV in artificial medium (pH 7,0) during heat treatment at 60,0 °C (a), 70,0 °C (b), 80,0 °C (c) and 90,0 °C (d). The straight lines are regression exponential curves

Inactivation curves at 60, 70, 80 and 90 °C are shown in figure 6. The inactivation curves fitted first order kinetics reasonably well, exept at 60 °C where the curve clearly seems to be bi-phasic. At 60 °C, approximately 85 % of infectivity was lost during the first minute of heat exposure. The inactivation rate then decreased abruptly and 1 Log_{10} reduction was encountered during the next 4 - 5 hours.

D-values were calculated from the formula for the regression exponential curves in Figure 6.

Table 8 Experimental D-values for IPNV in pH 7,0 artificial medium

Temperature	D-value (min)	R^2 value*
60 °C	287,82	0,35
70 °C	15,86	0,83
80 °C	2,74	0,74
90 °C	0,48	1,00

* R^2: Squared Correlation Coefficient

D-values were plotted against the corresponding exposure temperatures (Figure 7) and a z-value was calculated from the formula of the regression exponential curve. The D-value at 90 ºC was not included in z-value calculation because it was based on few datapoints and because infectivity was reduced to below detection limit before the target temperature was achieved. The Z-value of IPNV in pH 7,0 artificial medium was estimated to 9,9 °C.

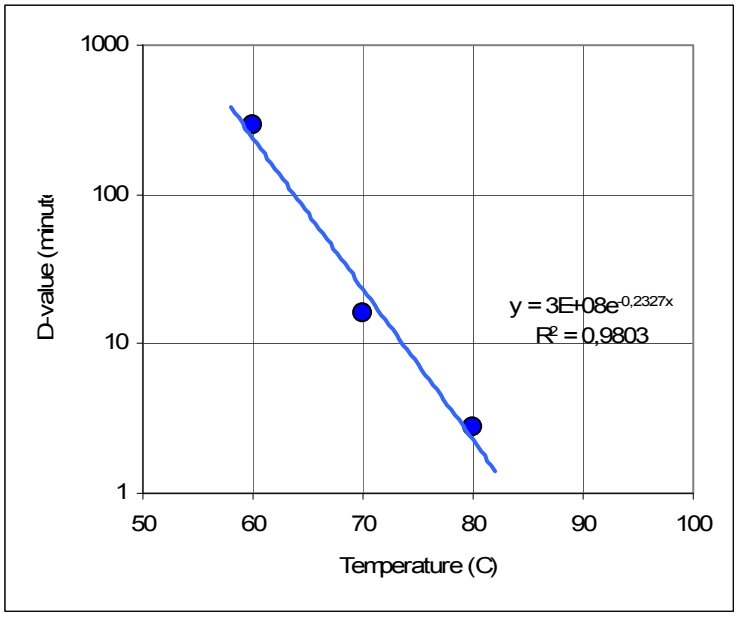
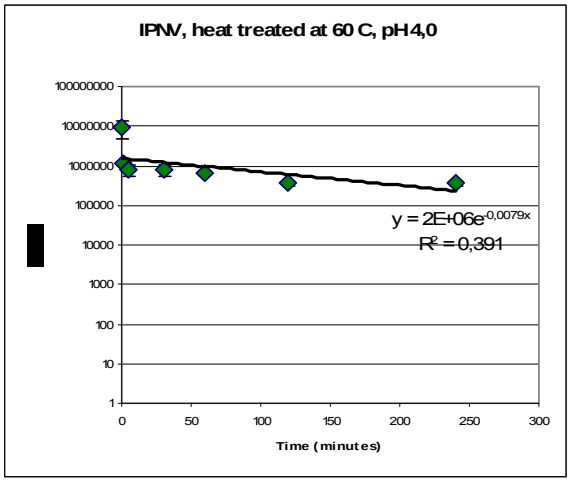


Figure 7 Log_{10} D-value versus exposure temperature for IPNV in pH 7,0 artificial medium. The straight line is a regression exponential curve. Line equation and the squared correlation coeffisient R^2 are shown in the figure.

3.6.3 Heat inactivation in artificial medium at pH 4,0

Artificial medium at pH 4,0 were inoculated 10:1 with an IPNV suspension containing approximately 6,8 Log_{10} $TCID_{50}$/ml before heat exposure.

a)



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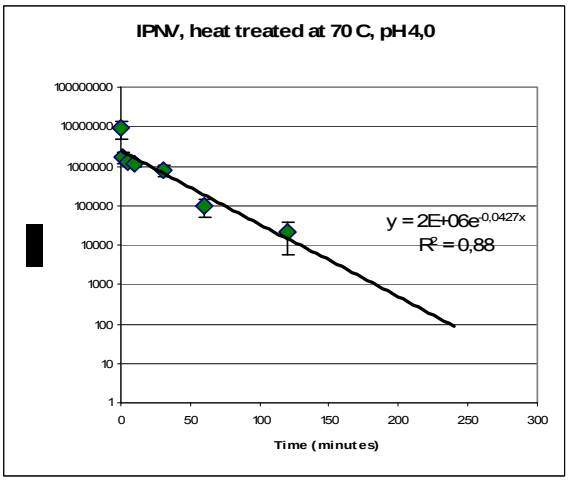
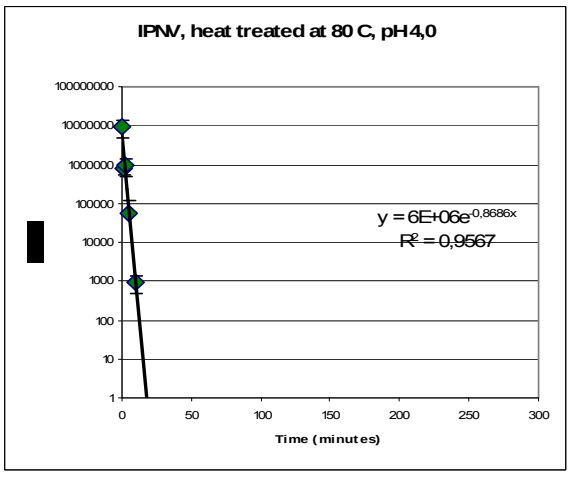


c)



d)

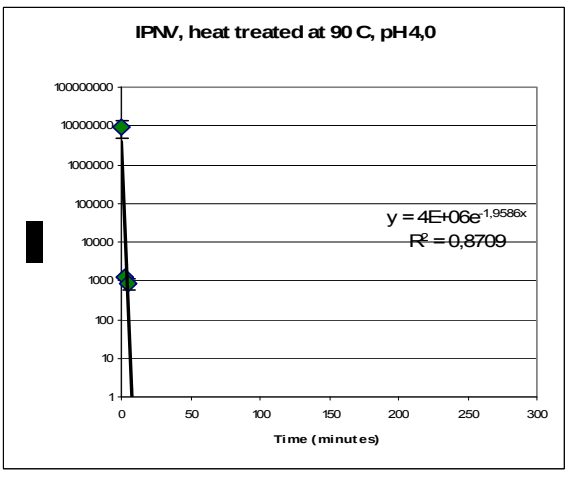


Figure 8 Inactivation curves for IPNV in artificial medium (pH 4,0) during heat treatment at 60,0 ºC (a), 70,0 ºC (b), 80,0 ºC (c) and 90,0 ºC (d). The straight lines are regression exponential curves. Each data point is the average of two parallell analyses. Vertical bars represent the standard deviation.

Inactivation curves at 60, 70, 80 and 90 ºC are shown in Figure 8. The inactivation curves fitted first order kinetics reasonably well, exept at 60 ºC where the data indicate a biphasic inactivation, similar to what was found after heat treatment at pH 7,0.

D-values were calculated from the formula for the regression exponential curves in Figure 8. The D-values obtained at pH 4,0 (Table 9) are quite similar to those obained at pH 7,0 (Table 8). However, in agreement with the results from the pre-investigation, most data points for the heat exposure in pH 4,0 media are approximately 1 log 10 higher than in pH 7,0 media.

Table 9 Experimental D-values for IPNV in pH 4,0 artificial medium

Temperature	D-value (min)	R^2 value*
60 °C	291,47	0,39
70 °C	53,92	0,88
80 °C	2,65	0,96
90 °C	1,17	0,88

* R^2: Squared Correlation Coefficient

D-values were plotted against the corresponding exposure temperatures (Figure 9) and a z-value was calculated from the formula of the regression exponential curve. As for the pH 7,0 experiments, the D-value at 90 ºC were not included in the z-value calculation because it was based on few datapoints and because infectivity was reduced to below detection limit before the target temperature was achieved. The Z-value of IPNV in pH 4,0 artificial medium was estimated to 9,8 °C.

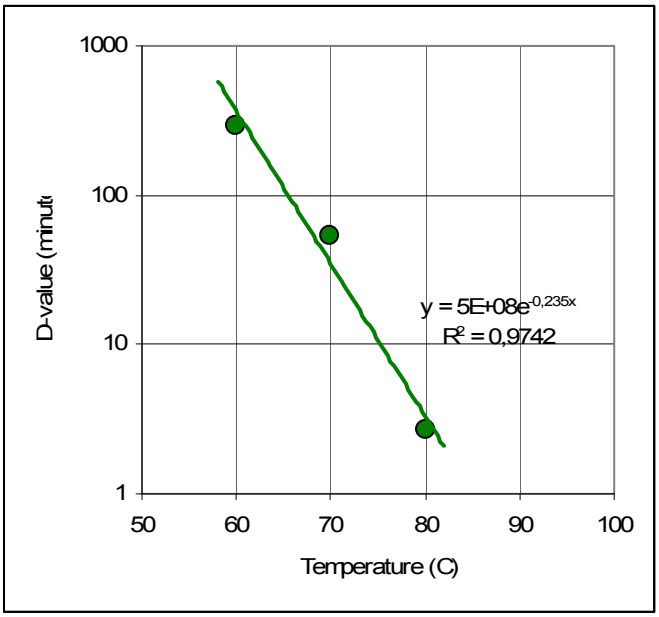


Figure 9 Log_{10} D-value versus exposure temperature for IPNV in pH 4,0 artificial medium. The straight line is a regression exponential curve. Line equation and the squared correlation coeffisient R^2 are shown in figure.

3.6.4 New processing method

Table 10 Concentration of infective IPNV (Log_{10} IPNV titer, $TCID_{50}$/ml) in γ-irradiated fish suspension after inoculation, cold storage, acid treatment and heat treatment. The fish suspension was inoculated 10:1 with an IPNV suspension containing approximately 7 Log_{10} $TCID_{50}$/ml. Two independent experiments were done. In the secound experiment, processing and analysis were carried out with two parallells.

	Exp. 1	Exp. 2a	Exp. 2b
	$Log_{10}TCID_{50}$/ml	$Log_{10}TCID_{50}$/ml	$Log_{10}TCID_{50}$/ml
Fish suspension prior to inoculation w. IPNV	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV and 24 hours cold storage	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment. Heat treated for 1 minute	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment. Heat treated for 2 minutes	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment. Heat treated for 5 minutes	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment. Heat treated for 10 minutes	< 3,8	< 3,8	< 3,8
After inoculation w. IPNV, 24 hours cold storage and 24 hours acid treatment. Heat treated for 25 minutes	< 3,8	< 3,8	< 3,8

In the first experiment with the new processing method (cold storage, acid treatment and heating) no infective IPNV could be recovered from the fish suspension at any step. Due to the unexpected results, the experiment was repeated in duplicate. The results confirmed that infective IPNV could not be detected after inoculation into the fish suspension.

4 Discussion

Industries processing fish by-products shall ensure that pathogens in the raw materials are adequately inactivated during the process. The effect of a temperature-time combination on a certain microorganism can be estimated, provided that the kinetic parameters (D- and z-values) for thermal inactivation of that organism are known. Unfortunately, few data on thermal resistance of viral fish pathogens have been published. The available data rarely contains D-values or data that can be used to estimate D-values. No z-values have been found.

IPNV is commonly regarded as the most heat resistant among fish-pathogenic micro-organisms (Defra, 2005, EC SCAHAW, 2003). However, few exact data on its temperature-dependant inactivation are available. Such data are therefore needed by the processing industry as well as by supervisory and regulatory authorities.

Earlier studies have demonstrated that thermal inactivation of IPNV is bi-phasic and that both phases follow 1. order kinetics. According to MacKelvie and Desaultes (1975), heat treatment at 60 ºC resulted in 3 Log_{10} reductions during the first 30 minutes and then 1 Log_{10} reduction per 80 minutes. In another study by Gousting and Gould (1981), 60 °C resulted in 1,5 Log_{10} reduction during the first 20 minutes and then 1 Log_{10} reduction per 160 minutes. Defra (2005) compared the inactivation effect on viral fish pathogens resulting from heating at 60 ºC for 1 and 24 hours. The results showed that IPNV was far more heat resistant than SVCV (D120), SVCV (880062), EHNV (sheatfish), EHNV (562/92) and CCV. It was also demonstrated that IPNV (serotype Ab) was more heat resistant than IPNV (serotype Sp) and IPNV (serotype 970160). The most resistant strain was reduced by 2,9 Log_{10} cycles in 24 hours. All inactivation data cited above refers to heat exposure in culture media with relatively low content of organic matter (10 % foetal bovine serum or less than 4 % bovine serum albumin). Inactivation data obtained at exposure temperatures higher than 60 ºC have not been published.

During the preparations for our own investigations, we planned to carry out heat exposure of IPNV in gamma irradiated (10 kGy) fish suspension. However, the initial examinations revealed that the fish suspension exhibited an adverse toxic effect on the BF-2 cell cultures, and had to be diluted at least 1:100 before virus analysis. This dilution seriously reduced the sensitivity of the analysis. Therefore, fish suspensions were replaced by artificial media in experiments intended for D- and z-value determination. Our artificial media contained 10,2 % soluble protein (Table 6) which is considerably more than in media used in the studies cited above. In general, high content of organic matter in the heating medium is considered to protect microorganisms against heat inactivation. This has also been shown for viruses (Alexander and Manvell, 2004). We suppose this may also be the case for IPNV, although we have found no data to support this generalization. Irrespective of this, we consider our artificial media, more than culture media, to resemble hydrolysed fish by-products and hence to be a more realistic matrix for our experiments.

In the present study, IPNV (serotype Sp) was heat treated in artificial media at pH 4 and 7. D-values (Table 8 and 9) obtained at 60 ºC was 4,8 hours irrespective of pH. At 70 ºC, D-values were 16 and 54 minutes in media with pH 7 and 4, respectively. At 80 ºC, D-values

were 2,7 minutes irrespective of pH. Both the pre-investigation (Table 7) and in the D-value determinations (Figure 6 and 7), showed that the infectivity of IPNV was better maintained at pH 4 than at pH 7.

The D-values found at 60 ºC agree reasonably well with the literature data referred above. For D-values obtained at 70, 80 and 90 ºC we find little basis for comparison in the scientific literature. In an investigation by Whipple and Rohovec (1994) the heating time needed to reduce IPNV concentration to below detection limit at 60, 70 and 80 ºC was 8 hours, 2 hours and 10 minutes, respectively. This is consistent with our results. In the same investigation, it was found that IPNV decreased more rapidly when heated at 65 ºC in neutral pH medium than when heated in pH 4,0 citric phosphate buffer. Smail et. al (1993) found that when a high dose of IPN (strain Sp) was used, 5 hours heating at 60 ºC in fish silage resulted in a titre reduction of 2,58 Log_{10}. Titre decreased by 2 Log_{10} cycles the first 2 hours of heat treatment and then by 0,5 Log_{10} cycle the next 3 hours. They also found that no infective virus was detectable (< 1 Log_{10} pfu/ml) after 1 hour when the initial concentration was 2-2,5 Log_{10} pfu/ml which was claimed to be typical for native silages. They concluded that 2 hours treatment of fish silage at 60 ºC would be sufficient for virus inactivation and would allow the silage to be used for feedstuff. The inactivation curve for IPNV heated at 60 ºC at pH 4 in our study seems to correspond well to the results of Smail et al.

Typical raw silages made from Atlantic salmon contain approximately 25 % of fat. As the fish material is minced and pumped during the manufacturing and storage of silage, it was of great interest to find out how IPNV was distributed between the immiscible water and oil phases. Our examination showed that the IPNV concentration after mixing of the phases was more than 10 times higher in water compared to the oil fraction. Therefore, migration of IPNV from water to oil should not be misinterpreted as IPNV inactivation when samples for virus analysis are drawn from the water phase of fish silage. Also, removal of the oily layer in the cell culture wells should not influence the virus titrations.

The regression exponential curves in Figure 7 and 9 (temperature vs. Log D-value) are reasonably parallel as can be seen from the z-values representing heat treatment at pH 4 and 7, respectively. It can be derived that the exposure temperatures must be 1,6 ºC higher at pH 4,0 than at pH 7,0 in order to achieve the same inactivation. For simplicity, the regression exponential curve obtained at pH 4,0 (Figure 9) can then be used to estimate minimum temperature-time combinations required in order to provide 2 or 3 Log_{10} reductions of IPNV at both pH values.

The regression exponential curves in Figure 6 and 8 are mainly determined by data from phase 2 of the bi-phasic inactivation curves. Therefore, when considering the total inactivation effect of a certain temperature-time combination, the rapid initial inactivation of approximately 0,7 Log_{10} reduction should also be added.

Table 11 Estimated exposure times required to achieve 1-5 Log_{10} reductions of infective IPNV by heat treatment in artificial medium. Total inactivation effects include the rapid initial inactivation (on average 0,7 Log_{10} reductions) and the subsequent exponential inactivation of phase 2.

Total inactivation effect	60 °C	65 °C	70 °C	75 °C	80 °C	85 °C	90 °C
1 Log_{10} reductions	102 min	32 min	10 min	3,0 min	0,9 min	0,3 min	0,1 min
2 Log_{10} reductions	441 min	137 min	42 min	13 min	4,0 min	1,2 min	0,4 min
3 Log_{10} reductions	781 min	242 min	75 min	23 min	7,1 min	2,2 min	0,7 min
4 Log_{10} reductions	1120 min	347 min	107 min	33 min	10 min	3,1 min	1,0 min
5 Log_{10} reductions	1460 min	452 min	140 min	43 min	13 min	4,1 min	1,3 min

Pathogens of fish may represent a hazard when materials from fish are used for feeding of fish. The heat treatment must therefore secure sufficient inactivation of such microorganisms. Regulation relating to the prohibition of using proteins of animal origin for production animals (FOR 2007-03-29 no 511), was laid down in Norway in 2007 pending Regulation (EC) 1774/2002 coming into force. In appendix 3, hygienization requirements were defined for materials from aquaculture fish used as feed for aquaculture fish; The method must be scientifically documented under adequate experimental conditions, to show minimum 3 Log_{10} (99,9 %) inactivation of *Aeromonas salmonicida,* subsp*. salmonicida* and IPN-virus. Appendix 3 was repealed in February 2009, after Regulation (EC) 1774/2002 had come into force in Norway. Still, it is a relevant reference to what can be considered as a sufficient inactivation effect for fish pathogens.

From Table 11 it can be found that a total of 3 Log_{10} reductions can be achieved by heat treatment at e.g. 75 °C for 23 minutes or 80 °C for 7,1 minute. The values in table 11 were estimated from the regression line for the relationship between D-values and temperatures at pH 4,0. As IPNV was more heat sensitive at pH 7 than at pH 4, we consider the values in table 11 to be conservative estimates valid for the pH range 7-4.

The results of our examinations confirm that IPNV is extremely heat resistant. The study also provides D- and z-values that can be used to predict the inactivation effect resulting from thermal processing of fish by-products at pH 4,0 (formic acid silage) and neutral pH. Such data has not been available before.

Based on the D- and z-values obtained during heat exposure of IPNV in artificial media, IPNV inactivation during the heating phase of the new processing method (85 ºC, 25 minutes) was calculated to 27 Log_{10} reductions. Even if IPNV is virtually unaffected by exposure to formic acid (pH 4), the heating phase alone will be fully sufficient to ensure adequate inactivation of IPNV during the complete process.

In the three experiments with the new processing method (cold storage, acid treatment and heating) no infective IPNV could be recovered from the fish suspension at any step. The results indicate that the fish suspension reduces the IPNV infectivity. Due to the toxic effect of the fish suspension to the cells, the sensitivity of the virus titration is reduced. With the given titer in the original virus suspension (7 Log_{10} $TCID_{50}$/ml), the experiments indicate a

reduction in IPNV infectivity ($TCID_{50}$) of 3 Log after inoculation into the fish suspension (and cold storage for approximately 48 hours) without any acid treatment and heating. A higher initial virus titer is needed to demonstrate a possible higher virus reduction. Compared to artificial medium, the fish suspension seems to contain a component that inactivates IPNV. Extended studies on other batches of fish suspensions should be performed before a general conclusion on the effect of fish suspensions can be made.

In appendix 3 of Regulation FOR 2007-03-29 no 511[2], the hygienization requirement for aquaculture fish used as feed for aquaculture fish was minimum 3 Log_{10} inactivation of IPNV. Appendix 3 was repealed in February 2009, after Regulation (EC) 1774/2002 had come into force in Norway. Still, it is a relevant reference to what can be considered as a sufficient inactivation effect for fish pathogens.

As the study indicates that IPNV is less stable at pH7 than at pH4, further studies should be performed on the effect of alkaline storage.

[2] FOR 2007-03-29 no 511, Regulation relating to the intra species recycling ban for animal protein

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