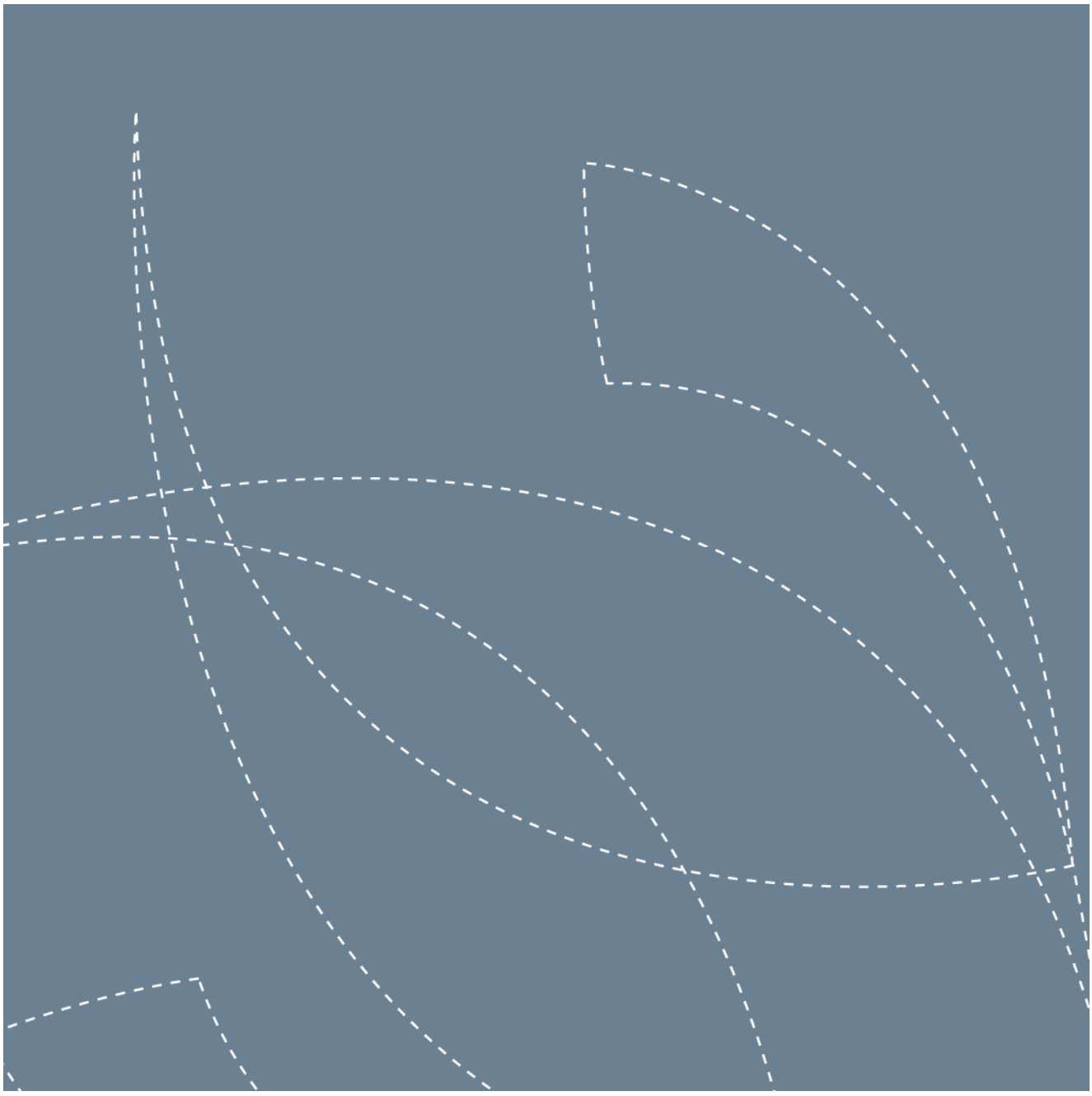
Report 22/2011 • Published May 2011

Inactivation of IPN-virus in fish by-products by inorganic acid and base

Halvor Nygaard, Nofima and Mette Myrmel, Veterinærinstituttet



Nofima is a business-oriented research group that aims at creating value with knowledge in close cooperation with the aquaculture-, fisheries- and food industry. The group has four research divisions: Marin, Mat, Ingrediens and Marked, around 470 employees altogether. Our headquarters are located in Tromsø. Other research units are at Ås, Stavanger, Bergen, Sunndalsøra and Averøy.

Ingredients Research specialises in research, analytical services and pilot production for the ingredients, aquaculture, food and pharmaceutical industries. Our core areas are raw materials competence, by- product utilisation, feeds and nutrition and processing of ingredients and feeds.

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

Nofima Marin AS
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
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

Report

ISBN: 978-82-7251-882-9 (printed)
ISBN: 978-82-7251-883-6 (pdf)

Report no: 22/2011

Accessibility: **Open**

Title:
Inactivation of IPN-virus in fish by-products by inorganic acid and base

Date:
24.05.2011

Number of pages and appendixes:
20

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

Project no.:
21123

By agreement with:
FHL and RUBIN

Contractors ref.:
G.H.Knutsen and S.Bekkevold

Three keywords:
Inorganic acid, inorganic base, IPN-virus

Summary:

The aim of the project was to find a simple, inexpensive and safe method for the treatment of category 2 fish by-products without heat treatment. IPNV was chosen as indicator organism because it was considered to be the hardiest among fish-pathogenic microorganisms. Salmonella and Clostridium perfringens were included in the study to provide a basis for assessing whether the method could also be applicable to category 3 by-products intended for feed production.

IPNV was inactivated (> 4 Log_{10} reductions) at pH below 1,5 or above 11,5. Depending on the composition of the by-products, this corresponds to dosage of 1,0-1,8 % HCl or 0,9-2,0 % KOH.

Salmonella was inactivated at pH between 3,0 and 4,0 or above 11,0. This corresponds to less than 1,0 % HCl, or, depending on the composition of the by-products, 0,7-1,7 % KOH. Salmonella is therefore securely inactivated at conditions required to control IPNV.

HCl treatment for 5 hours at pH 1,0 gave 1 Log_{10} reduction of viable C.perfringens spores. No inactivation was observed at high pH values, up to 12,0. The pH conditions required to control IPNV does not affect the viability of C.perfringens spores.

The present study shows that the required inactivation of IPNV in fish by-products can be achieved by treatment with both inorganic acid and base. The effect of these treatments on processability and as product properties in relation to intended utilizations should be further examined.

Table of contents

1 Introduction

The aquaculture industry needs a simple, inexpensive and safe method for treatment of fish by-products without heat treatment. The Norwegian Seafood Federation (FHL) therefore initiated a project to determine the inactivation effect on infectious pancreas necrosis virus (IPNV) by treatment of fish by-products with inorganic acid or base.

The purpose of the investigations was to provide documentation as basis for an application for approval of a new method for treatment of category 2 fish by-products as described in Regulation (EC) 1774/2002. The method could replace an unnecessary, costly and energy-intensive heat treatment of fish by-products to be used for bio-energy production. Increased opportunities for local bio-energy, fertilizer or soil improver industries could also be provided. Approval of a simplified method would fullfill the objective that regulations should be proportionate in relation to the hazards to be controlled. It would also contribute to a more sustainable production by reducing energy use and CO_2 emission.

The method should also be considered approved as an alternative to energy-intensive heat treatment of category 3 fish by-products intended as feed ingredient for farmed fish and warm-blooded production animals (except ruminants). This could increase the exploitation of by-products as feedstuffs and the industry would get more opportunities for local processing without transport to central heat treatment plants.

IPNV was chosen as indicator organism because it is considered to be the most hardy among fish-pathogenic microorganisms. (Schei and Torgersen, 1990, Christie and Hjeltnes, 1990, EC SCAHAW, 2003). It was assumed that a treatment which inactivates IPNV in fish by-products would also inactivate other infectious agents present.

In a previous project (Rubin report no. 199), it was found that fish suspensions had a toxic effect on the BF-2 cells used for quantification of infective IPNV. Therefore, an artificial medium with similar content of water soluble protein and salt was developed. This medium was used in inactivation experiments where a high sensitivity was required. A natural matrix, gamma sterilized fish suspension, was used for final verification of the results. The same strategy and the same media were used in the present study.

In Regulation (EC) 1774/2002, *Salmonella*, *Enterobacteriaceae* and *Clostridium perfringens* are used as indicators for the hygienization effect of alternative processing methods. These organisms were therefore included in the study in order to provide a basis for assessing whether the method could also be approved for treatment of category 3 by-products.

2 Materials and methods

2.1 Preparation of gamma sterilized fish suspension

Head/backbones from Atlantic salmon and sea trout were coarsely minced and subsequently mixed with equal parts of viscera before repeated 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 frozen to Institute for Energy Technology (N-2027 Kjeller, Norway) and exposed to 10 kGy of gamma irradiation to inactivate the indigenous microbial flora. After irradiation, the fish suspension was stored frozen until use.

The same suspension was used as a heating matrix in earlier studies on *Salmonella* (Rubin report no 180), IPN-virus (Rubin report no 199) and *Clostridium sporogenes* spores (Rubin report no 203).

2.2 Preparation of fish suspensions from by-product fractions

Whole fish, head/backbones and viscera from Atlantic salmon were separately minced in a meat grinder with coarse screen (11 mm). The minces were further homogenized using an Ultra-Turrax knife homogenizer. The homogenates were distributed in 500 ml capacity screw capped polyethylene bottles and frozen at ≤ -20 °C. The suspensions were used in pH-titration experiments to determine acid- or base expenditure.

2.3 Preparation of artificial medium

Artificial medium with content of water soluble protein and salt as in raw fish silage was prepared from bacteriological peptone, Oxoid L 37 (121 g/l) and NaCl (2 g/l). The medium was sterilized at 121 °C for 15 minutes.

2.4 Preparation of test microorganisms

2.4.1 IPN-virus

IPN-virus (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 50 µg/ml Gentamicin. Three rounds of virus propagation were performed, giving virus titers of approximately 6-7 Log_{10} $TCID_{50}$/ml. The virus suspension was stored at 4 ºC until use.

2.4.2 Salmonella

A freeze-dried culture of *Salmonella enterica subspecies enterica serovar senftenberg* (CCUG 19369) was purchased from Culture Collection, University of Gothenburg, Sweden. The content of one vial was reconstituted in peptone-salt solution (ISO 6887-1), spread on

NA (Nutrient Agar, Oxoid CM 0003) and incubated at 37 °C over night. One pure colony from NA was transferred to NB (Nutrient Broth, Oxoid CM 0001) and incubated at 37 °C over night. The NB culture (100 ml) was finally mixed with 20 ml of 60 % autoclaved glycerol, distributed in cryo-tubes (Nunc 363401) and freeze-stored at ≤ -20 °C.

Fresh cultures (late exponential phase) were prepared by transferring some ice crystals from the frozen stock-cultures to NB and incubated at 37 °C for 16-18 hours.

2.4.3 Clostridium perfringens

Freeze dried tablets of *C.perfringens* (BioBall, High dose 10K) each containing 8,2 x 10^3 spores of *C.perfringens* (NCTC 8798), were purchased from BTF (Sydney, Australia).

2.5 Quantification of microorganisms

2.5.1 IPN-virus

Infectious IPNV was quantified by titration in BF-2 cells (without FBS) in 96 well plates with 6 parallels of each dilution. After 90 min of incubation, the virus samples were removed from the cells and medium was added. The cells were screened for cytopathogenic effect after 7 days. Viral titer was determined by the method of Kärber (1979). Titration was performed in duplicates.

2.5.2 Salmonella

Salmonella was quantified by the MPN (most-probable-number) technique (U.S. FDA, 2006).

The initial suspension (10^{-1} dilution of the sample) and further ten-fold dilutions were prepared according to ISO 6887-1. Preenrichment cultures were started in triplicates by transferring 1 ml portions of each diluted sample to 9 ml buffered peptone water (Merck 1.07228). After incubation for 20 hours at 37 °C, 1 ml was subcultured in 9 ml brain heart infusion (Merck 1.10493) for 3 hours at 37 °C.

Salmonella was detected in the BHI cultures by Real Time PCR analysis (Biotecon Diagnostics, Foodproof *Salmonella* Detection Kit, R30027) after DNA extraction (Biotecon Diagnostics, Foodproof *Salmonella* ShortPrep Kit, S40001). The ratio of positive results to negative results in relation to the dilution rate results in a MPN/g value.

2.5.3 Clostridium perfringens

C.perfringens was analysed according to ISO 7937.

Initial suspensions (10^{-1} dilution of the sample) and further ten-fold dilutions were prepared according to ISO 6887-1.

1 ml portions of each dilution were mixed with liquefied TSC-agar (Oxoid CM 0587) in 9 cm Petri-dishes and allowed to solidify prior to incubation. The detection limit was lowered to 1

CFU gram^{-1} by additional plating of 10 ml portions of the undiluted sample in 13 cm Perti-dishes.

Plates were incubated in a modified atmosphere jar (GasPak 150 System, BBL). Anaerobic conditions were created using Anaerogen Sachets (Oxoid AN 0025) and controlled by Dry Anaerobic Indicator Strips (BBL no. 271051). The jars were incubated at 37 ºC for 24 hours. After incubation, typical colonies were counted. Presumptive *C.perfringens* form black colonies in TSC-agar. Characteristic colonies were confirmed by LS-medium.

The concentration of organisms in the samples was calculated as weighted mean of the counts from two successive dilutions according to ISO 7218.

2.6 pH-titrations

Portions (100 ml) of minced whole salmon, head/backbone fraction and viscera fraction were titrated with 10 M solutions of HCl and KOH.

2.7 Treatment of microorganisms with inorganic acid or base

2.7.1 IPN-virus

A preinvestigation was conducted to determine proper acid- or base concentrations and treatment times to be used in the main experiment.

Artificial medium was inoculated with IPNV suspension (11:1). Portions of 3,3 ml was pH adjusted and incubated at 20-22 ºC. In the preinvestigation, the medium was neutralized after 5 hour (Table 1), while in the main experiment medium was neutralized after 4, 8 or 24 hours (Table 2 and 3). Finally, the medium was transferred to sterile plastic tubes and kept at 0-5 ºC until analysis. A sample of the IPNV suspension was kept untreated at 4 ºC and co-analysed with the pH adjusted samples.

IPNV inoculated into irradiated fish suspension was treated as described in Table 4.

Table 1 Preinvestigation, treatment of IPNV in artificial medium (1:11 in a total of 3,3 ml)with HCl or KOH at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
pH 1,0	0,144		0,150		5,0
pH 2,0	0,099		0,103		5,0
pH 3,0	0,060		0,062		5,0
pH 4,0	0,027		0,028		5,0
pH 6,1					5,0
pH 9,0		0,038		0,034	5,0
pH 10,0		0,063		0,057	5,0
pH 11,0		0,087		0,079	5,0
pH 12,0		0,101		0,091	5,0

Table 2 Main experiment. Treatment of IPNV in artificial medium (1:11 in a total of 3,3 ml) with HCl at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
pH 1,0	0,144		0,151		4,0
	0,144		0,151		8,0
	0,144		0,151		24,0
pH 1,5	0,117		0,123		4,0
	0,117		0,123		8,0
	0,117		0,123		24,0
pH 2,0	0,099		0,104		4,0
	0,099		0,104		8,0
	0,099		0,104		24,0
pH 2,5	0,081		0,085		4,0
	0,081		0,085		8,0
	0,081		0,085		24,0
pH 3,0	0,060		0,062		4,0
	0,060		0,062		8,0
	0,060		0,062		24,0
NEG. CONTROL	0,144		0,151		24,0

Table 3 Main experiment. Treatment of IPNV in artificial medium (1:11 in a total of 3,3 ml) with NaOH at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
pH 10,0		0,063		0,056	4,0
		0,063		0,056	8,0
		0,063		0,056	24,0
pH 10,5		0,078		0,070	4,0
		0,078		0,070	8,0
		0,078		0,070	24,0
pH 11,0		0,090		0,081	4,0
		0,090		0,081	8,0
		0,090		0,081	24,0
pH 11,5		0,097		0,087	4,0
		0,097		0,087	8,0
		0,097		0,087	24,0
pH 12,0		0,102		0,092	4,0
		0,102		0,092	8,0
		0,102		0,092	24,0
NEG. CONTROL		0,102		0,092	24,0

Table 4 Treatment of IPNV in gamma sterilized fish suspension (1:11 in a total of 11,0 ml) with HCl or KOH at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
1,5	0,28		0,28		24,0
1,5	0,28		0,28		24,0
NEG. CONTROL	0,28		0,28		24,0
11,0		0,52		0,52	24,0
11,0		0,52		0,52	24,0
NEG. CONTROL		0,52		0,52	24,0

2.7.2 Salmonella

Gamma sterilized fish suspension was inoculated with a diluted late exponential phase culture of *Salmonella* (2,8 x 10^7 /ml). Inoculated medium was pH adjusted (Table 5) and incubated at 20-22 ºC. The medium was neutralized after 5 hour before quantification of viable *Salmonella*.

Table 5 Treatment of Salmonella in gamma sterilized fish suspension (1:200 in a total of 10,0 ml) with HCl or KOH at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
pH 1,0	0,33		0,33		5,0
pH 2,0	0,22		0,22		5,0
pH 3,0	*0,15*		*0,15*		*5,0*
pH 4,0	0,08		0,08		5,0
pH 6,3					5,0
pH 9,0		0,32		0,32	5,0
pH 10,0		0,43		0,43	5,0
pH 11,0		0,52		0,52	5,0
pH 12,0		0,60		0,60	5,0

2.7.3 Clostridium perfringens

A *C.perfringens* spore suspension was prepared by dissolving 10 BioBall High Dose 10K tablets in 10 ml distilled water.

Gamma sterilized fish suspension was inoculated with the freshly prepared *C.perfringens* spore suspension (7,3 x 10^3 /ml). Inoculated medium was pH adjusted (Table 6) and incubated at 20-22 ºC. The medium was neutralized after 5 hour before quantification of viable *C.perfringens* spores.

Table 6 Treatment of C.perfringens spores in gamma sterilized fish suspension (1:11 in a total of 11,0 ml) with HCl or KOH at 20-22 ºC.

	pH adjustment		pH neutralization		
	10 M HCl	10 M KOH	10 M KOH	10 M HCl	Time
Target pH	(ml)	(ml)	(ml)	(ml)	(hours)
pH 1,0	0,33		0,33		5,0
pH 2,0	0,22		0,22		5,0
pH 3,0	0,15		0,15		5,0
pH 4,0	0,08		0,08		5,0
pH 6,3					5,0
pH 9,0		0,32		0,32	5,0
pH 10,0		0,43		0,43	5,0
pH 11,0		0,52		0,52	5,0
pH 12,0		0,60		0,60	5,0

3 Results and discussion

3.1 Characterization of fish suspensions

Table 7 Main constituents of fish suspensions.

Fish species Fraction		Salmon Whole fish	Salmon Head/backbone	Salmon Viscera	Salmon, trout Head/backbone, viscera
Homogenized		+	+	+	+
Sieved		-	-	-	+
Gamma sterilized		-	-	-	+
Raw protein	(ISO 5983)	17,5 %	14,7 %	5,5 %	6,6 %
Dry matter	(ISO 6496)	43,0 %	44,1 %	58,5 %	60,2 %
Ash	(ISO 5984)	2,1 %	3,4 %	0,6 %	0,8 %
Fat	(NS 9402)	22,8 %	26,2 %	52,7 %	54,4 %

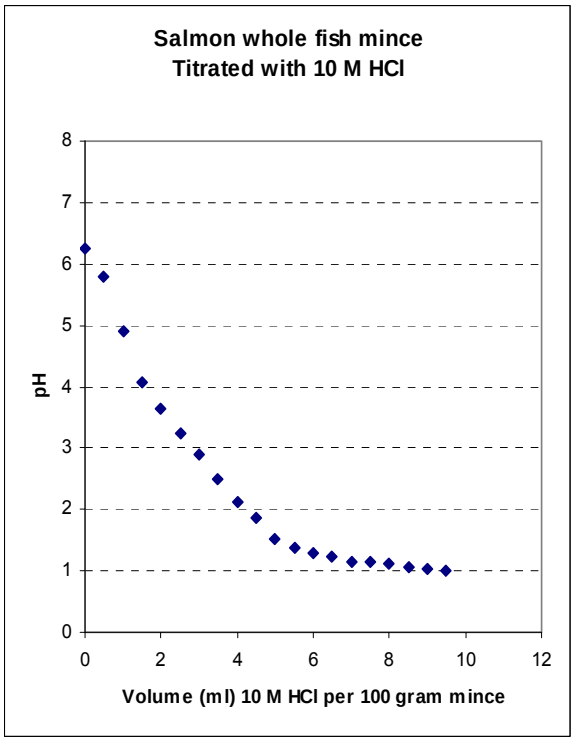
The shape of the titration curves are determined by the gross composition (fat, protein, ash and water) of the fish material and by the state of those constituents.

High fat content will contribute to steep titration curves because added acid or base are distributed in a relatively small aqueous phase. Protein and ash have buffering capability and will contribute to flat titration curves.

The buffering capacity of proteins increase with degree of hydrolysis; i.e., the percentage of hydrolyzed peptide bonds. Proteolytic enzymes from the fish gastrointestinal tract digest fish proteins in the homogenized suspensions. Therefore, fish suspensions containing viscera tend to be rapidly hydrolysed.

Ash is mainly hydroxyapatite ($Ca_5(PO_4)_3(OH)$) and $Ca(CO_3)_2$ from fish bones, and the buffering capacity increase with the degree of mineral dissolution. Acid treatment dissolves bone minerals; therefore titration with acid will result in increased buffering capability of suspensions containing fish bones.

a)



b)

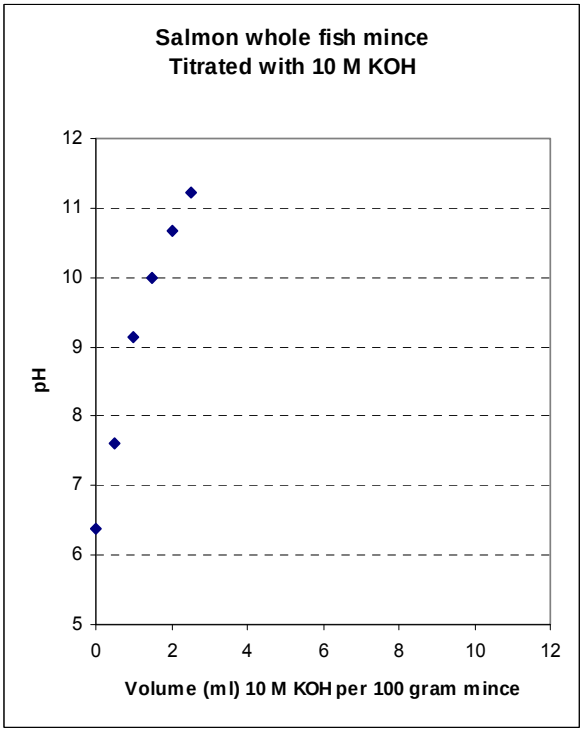
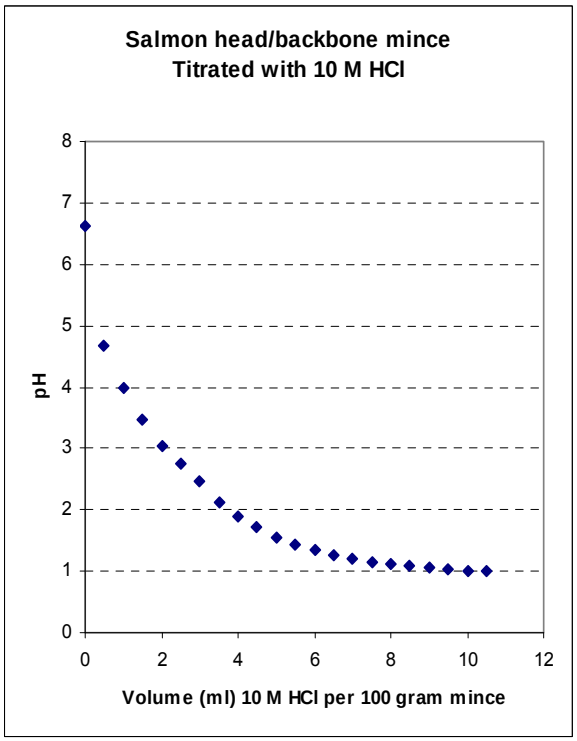


Figure 1 pH of minced whole salmon versus amount of added 10 M HCl (a) or 10 M KOH (b).

a)



b)

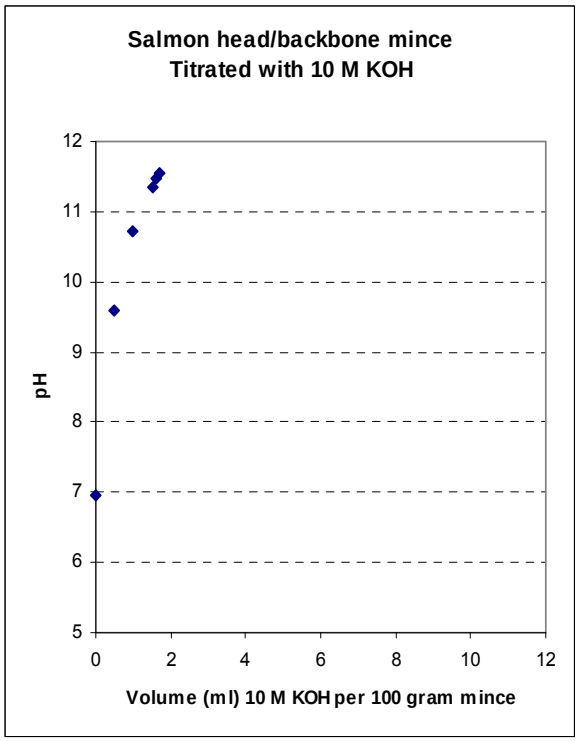
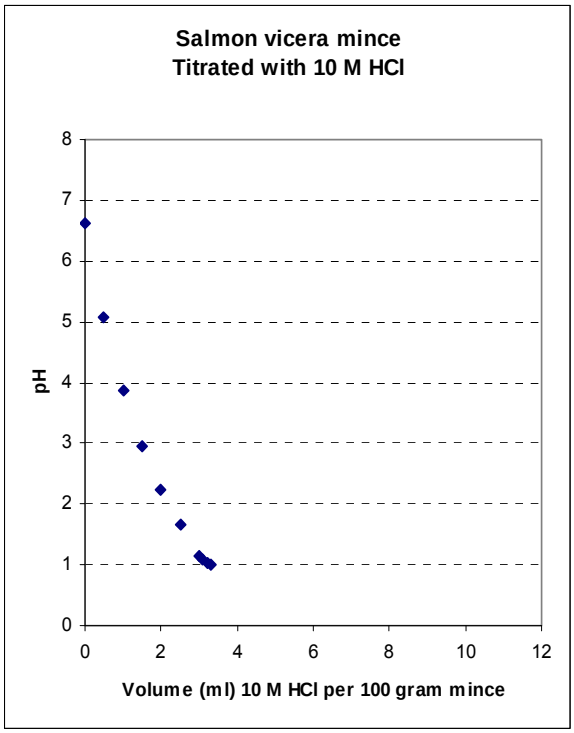


Figure 2 pH of minced salmon head/backbones versus amount of added 10 M HCl (a) or 10 M KOH (b).

a)



b)

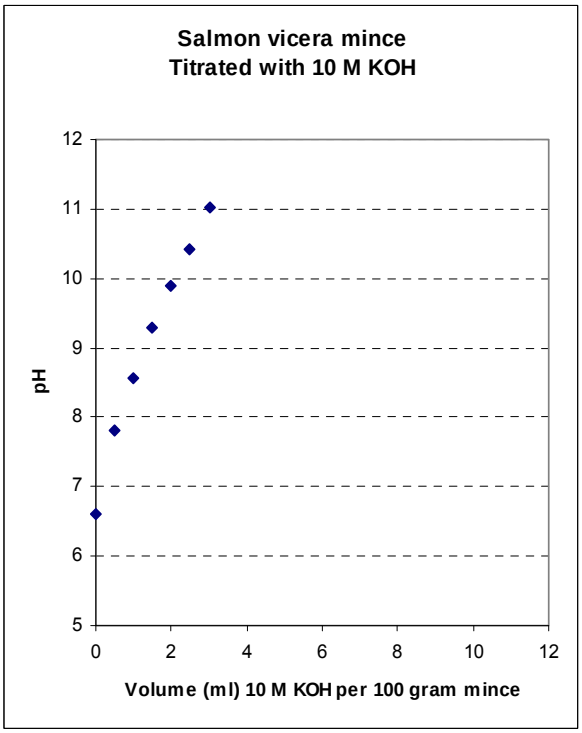


Figure 3 pH of minced salmon viscera versus amount of added 10 M HCl (a) or 10 M KOH (b).

3.2 Characterization of artificial medium

According to analysis, the artificial medium contained 10,2 % protein and 0,3 % salt, which corresponds well to the typical composition of commercial fish silages; 10,6 % protein and 0,4 % salt in the water soluble fraction (data on the composition of fish silages was provided by Hordafor AS).

The artificial medium had pH 6,1 after sterilization, a deep brown colour and was free of haze and precipitates.

a) b)

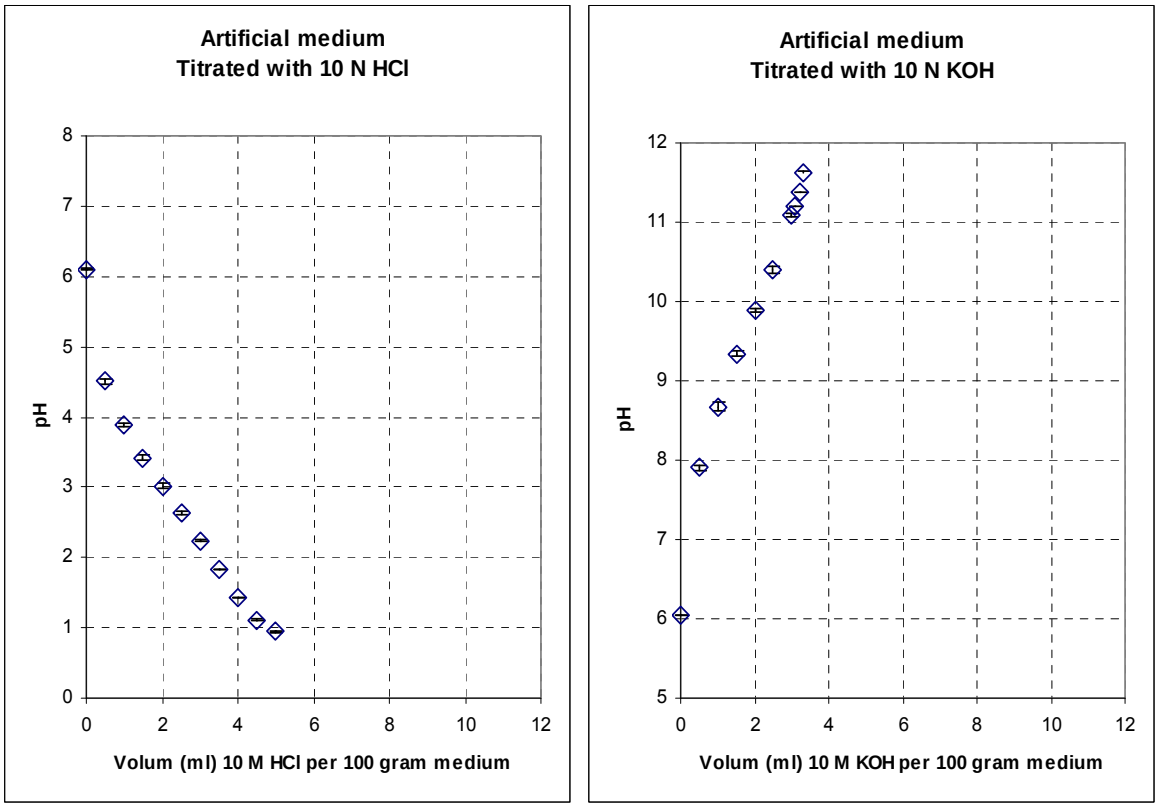


Figure 4 pH of artificial medium versus amount of added 10 M HCl (a) or 10 M KOH (b).

3.3 Expenditure of acid and base

The expenditure of HCl and KOH required to reach specified target pH levels in salmon by-products was derived from the titration curves (Figure 1, 2, 3). The amount of acid or base is mainly determined by the gross composition of the fish by-products, the degree of protein hydrolysis and the dissolution rate of bone minerals.

Table 8 shows the amount of acid or base required to reach different pH levels. The values can be basis for cost estimates.

Table 8 Concentration of HCl (a) and KOH (b) required to reach specified target pH levels in different fish suspensions.

a) HCl

	HCl (%, w/v)		
Target pH	Whole fish mince	Head/backbone mince	Viscera mince
3,0	1,02	0,77	0,51
2,5	1,28	1,10	0,66
2,0	1,53	1,35	0,80
1,5	1,83	1,93	0,99
1,0	3,47	3,65	1,13

b) KOH

	KOH (%, w/v)		
Target pH	Whole fish mince	Head/backbone mince	Viscera mince
10,0	0,84	0,34	1,23
10,5	1,06	0,50	1,46
11,0	1,29	0,67	1,68
11,5	1,51	0,90	1,96
12,0	~ 1,90	~ 1,46	~ 2,35

3.4 Inactivation of microorganisms

3.4.1 IPN-virus

Table 9 Pre-investigation. Concentration of infective IPN-virus (Log_{10} $TCID_{50}$/ml) in artificial medium after exposure to different dosages of HCl and KOH.

Treatment		Log $TCID_{50}$/ml 5 hours
pH 1,0	HCl	< 1,8
pH 2,0	HCl	6,1
pH 3,0	HCl	6,3
pH 4,0	HCl	6,3
pH 6,1	untreated	5,1
pH 9,0	KOH	5,0
pH 10,0	KOH	4,5
pH 11,0	KOH	4,1
pH 12,0	KOH	< 1,8

The results (Table 9) show that exposure to HCl and KOH had little effect on the infectivity of IPNV unless pH was below 2,0 or above 11,0, respectively.

For pH 2-4 the virus titre is higher than in the untreated virus suspension (pH 6,1). This was also observed in previous experiments with formic acid treatment of IPNV (RUBIN, 2010) and indicates that acid treatment of IPNV induces an increase of virus infectivity which is not reversed by neutralization. However, this was not found in the main experiment (Table 10a) and repetitions must be performed to bring this to a conclusion.

The exposure times and the pH levels to be used in the main experiments were based on the results in the pre-investigation.

Table 10 Concentration of infective IPN-virus (Log_{10} $TCID_{50}$/ml) in artificial medium after exposure to different dosages of HCl and KOH.

a) Main experiment

		Log $TCID_{50}$/ml		
Treatment		4 hours	8 hours	24 hours
pH 1,0	HCl	< 1,8	< 1,8	< 1,8
pH 1,5	HCl	3,6 ± 0,0	3,1 ± 0,4	3,7 ± 0,1
pH 2,0	HCl	5,1 ± 0,1	4,9 ± 0,4	4,7 ± 0,6
pH 2,5	HCl	5,6 ± 0,1	5,7 ± 0,2	5,3 ± 0,3
pH 3,0	HCl	5,9 ± 0,1	5,8 ± 0,4	4,7 ± 0,2
pH 10,0	KOH	4,1 ± 0,1	5,1 ± 0,4	6,2 ± 0,2
pH 10,5	KOH	3,5 ± 0,2	5,8 ± 0,0	5,8 ± 0,3
pH 11,0	KOH	3,3 ± 0,3	4,9 ± 0,1	5,3 ± 0,0
pH 11,5	KOH	< 1,8	2,2 ± 0,2	3,8 ± 0,3
pH 12,0	KOH	< 1,8	< 1,8	< 1,8

b) Repetition of 4 and 24 hours incubation at pH 10,5 and 11,0.

		Log $TCID_{50}$/ml		
Treatment		4 hours	8 hours	24 hours
pH 6,1	untreated			6,1 ± 0,0
pH 10,5	KOH	5,3 ± 0,0		5,6 ± 0,1
pH 11,0	KOH	5,2 ± 0,1		5,3 ± 0,0

Table 11 Concentration of infective IPN-virus (Log_{10} $TCID_{50}$/ml) in gamma sterilized fish suspension after exposure to selected dosages of HCl and KOH.

		Log $TCID_{50}$/ml		
Treatment		4 hours	8 hours	24 hours
pH 1,5 (A)	HCl			< 3,8
pH 1,5 (B)	HCl			< 3,8
pH 11,0 (A)	KOH			5,4 ± 0,1
pH 11,0 (B)	KOH			5,1 ± 0,0

The main experiment (Table 10a) shows that acid treatment causes considerable inactivation at pH ≤ 1,5 during the first 4 hours of incubation but little additional effect by further incubation. Base treatment at pH ≥ 10,5 for 4 hours gave similar inactivation, but surprisingly the effect was reversed during prolonged exposure. When parts of the experiment with base treatment were repeated (Table 10b), the trend was confirmed although the effect was less pronounced and could be due to variations in titration.

However, the 24 hours titre in gamma sterilized fish suspension (pH 11,0) support the possible time dependent effect of pH 10,0-11,5 to IPNV. In a former experiment (RUBIN 2010), no infective IPNV could be found after inoculation into this fish suspension when there was no pH regulation. A possible explanation is that IPNV neutralizing components in the fish suspension are denatured by high pH and that infective IPNV thereby is released.

The results from 4 hours of acid/base treatment correspond well to the results of the pre-investigation (5 hours). Furthermore, the results from the repeated 24 hours treatment with base and from acid and base treatment of gamma sterilized fish suspension (Table 11) correspond well with the results from the main experiment.

In total, the experiments show that > 4 Log_{10} inactivation of IPNV with inorganic acids or bases requires pH below 1,5 or above 11,5. The results of our study are in agreement with previous reports.

According to Defra (2005), an artificial medium adjusted to pH 12,0 with NaOH gave 100 % inactivation of IPNV serotype Sp, Ab and 970160 within respectively 20, 60 and 60 minutes.

According to Rubin (2003), adjustment to pH 12,0 in finely minced fish resulted in > 3 Log_{10} reductions within 16 hours while adjustment to pH 11 gave approximately 2 Log_{10} reductions within 24 hours. Adjustment to pH 10 resulted in 1,3 Log_{10} reductions during the first 16 hours, followed by 1,1 Log_{10} increase the next 8 hours, indicating a time dependant effect of pH 10 to IPNV infectivity.

According to Ahne (1984), adjustment to pH 11,9 with NaOH resulted in 100 % inactivation within 5 minutes. Adjustment to 3,0 with HCl had no effect within 60 minutes.

3.4.2 Salmonella

Table 12 Concentration of Salmonella (Log MPN/ml) in gamma sterilized fish suspension after exposure to different dosages of HCl and KOH. Initial concentration was 140.000/ml (Log MPN/ml = 5,1).

Treatment		Log MPN/ml 5 hours
pH 1,0	HCl	< 0,0
pH 2,0	HCl	< 0,0
pH 3,0	HCl	< 0,0
pH 4,0	HCl	5,0
pH 6,3	untreated	> 5,4
pH 9,0	KOH	5,0
pH 10,0	KOH	4,0
pH 11,0	KOH	0,6
pH 12,0	KOH	< 0,0

The results (Table 12) show that *Salmonella* multiplies in fish suspension with no added HCl or KOH. HCl treatment at pH 4 for 5 hours resulted in impaired growth but no inactivation. At pH 3,0 or below, *Salmonella* underwent more than 5 Log_{10} reductions. Addition of KOH to pH 9,0 resulted in impaired growth but no inactivation. KOH addition to pH 10,0 and 11,0 resulted in, respectively, 1 and more than 4 Log_{10} reductions.

The experiments show that complete *Salmonella* inactivation with inorganic acids or bases require pH below 4,0 or above 11,0.

We consider the family *Enterobacteriaceae* to be fairly uniform with regard to acid tolerance, and presume that the results found for *Salmonella* are valid for the group in general.

3.4.3 Clostridium perfringens

The properties of the spores in BioBall High Dose 10K tablets were examined prior to use in inactivation experiments. Viable spores in each tablet were enumerated before and after heat activation at 80 ºC for 10 minutes. Approximately 8000 bacteria were found in both cases, showing that the preparation contains no vegetative bacteria and that the spores germinate in TSC-agar without heat activation.

Table 13 Concentration of viable C.perfringens spores (Log cfu/ml) in gamma sterilized fish suspension after exposure to different dosages of HCl and KOH. Initial spore concentration was 730/ml (Log cfu/ml=2,8). The results are average of two parallel analyses.

Treatment		Log cfu/ml 5 hours
pH 1,0	HCl	1,8
pH 2,0	HCl	2,7
pH 3,0	HCl	2,8
pH 4,0	HCl	2,7
pH 6,3	untreated	2,7
pH 9,0	KOH	2,8
pH 10,0	KOH	2,6
pH 11,0	KOH	2,7
pH 12,0	KOH	2,5

The results (Table 13) show that HCl treatment for 5 hours at pH 1 gave 1 Log_{10} reduction of viable *C.perfringens* spores. No inactivation was observed at higher pH values, up to 12,0.

The experiments show that complete inactivation of *C.perfringens* spores with inorganic acids or bases require pH below 1,0 or above 12,0.

4 References

Ahne W (1984) Vergleichende Untersuchungen über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV) Zbl. Vet. Med. B 1982 (29): 457-476.

Christie, K.E. og Hjeltnes, B. (1990) Infeksiøs Pankreas Nekrose-IPN. In: Fiskehelse-Sykdommer, forebygging, behandling. Red. T.Poppe. John Grieg Forlag AS.

Defra (2005) Research project final report, SID 5 Inactivation of fish pathogens following ensiling or composting.

EC SCAHAW (2003) The use of fish by-products in aquaculture Report of the Scientific Committee on Animal Health and Animal Welfare European Commission, Health and Consumer Protection Directorate General.

ISO 6887-1. Microbiology - General rules for the preparation of dilutions for microbiological examinations.

ISO 7218. Microbiology of food and feeding stuffs - General rules for microbiological examinations.

ISO 7937. Microbiology of food and animal feedingstuffs - Horizontal method for the enumeration of *Clostridium perfringens* - Colony count technique.

Kärber G. (1979) Calculation of the LD_{50} titer by the Kärber method. In: Lennette E.H., Schmidt N.J. (Eds.), Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, 5th edition, American Public Health Association, Washington, D.C., pp. 34-35.

Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3rd October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Rubin (2003) Report no 4502/107 Hygieniserende effekt av Gellyfeed prosessen.

Rubin (2009) Report no. 180 Inaktivering av patogene mikroorganismer I fiskebiprodukter. Delprosjekt *Salmonella.*

Rubin (2010) Report no. 199 Inaktivering av patogene mikroorganismer I fiskebiprodukter. Delprosjekt IPN-virus.

Rubin (2011) Report no. 203 Inaktivering av patogene mikroorganismer I fiskebiprodukter ved ensilering og varmebehandling. Delprosjekt Clostridium sporogenes sporer.

Schei, I og Torgersen, Y.A. (1990) Behandling av blodvann og forsøk med fiskepatogener. Havbruk nr. 7/1990.

U.S. FDA. Center for Food Safety and Applied Nutrition (2006). Bacteriological Analytical Manual Online, Appendix 2: Most Probable Number from Serial Dilutions. Available at: http://vm.cfsan.fda.gov/~ebam/bam-a2.html

.

ISBN 978-82-7251-882-9 (printed)
ISBN 978-82-7251-883-6 (pdf)
ISSN 1890-579X