Report

QLK2-CT-2002-01546: **Fish Egg Trade**

Work package 3 report: Pathogen survival outside the host, and susceptibility to disinfection

VESO VETERINARY SCIENCE OPPORTUNITIES
MINISTRY OF FAMILY AND CONSUMER AFFAIRS
Danish Institute for Food and Veterinary Research
Cefas
INRA
Veterinærinstituttet National Veterinary Institute
IZS
AquaGen

Impressum

Title:
Work package 3 report: Pathogen survival outside the host, and susceptibility to disinfection

Authors:
Giuseppe Bovo, Barry Hill, Asbjørn Husby, Tore Håstein, Christian Michel, Niels J. Olesen, Arne Storset and Paul J. Midtlyng

Issued by: Veterinærmedisinsk Oppdragssenter AS	**Sponsor:** European Commission
Project number: VESO-1601	**Sponsor´s reference:** Contract no QLK2-CT-2002-01546
Project manager: Tania Wolffrom (until March 2004), Paul J. Midtlyng (from April 2004)	**Contact person:** Isabel Minguez-Tudela

Date: August 20, 2005	**Availability:** open ISBN: 82-91743-36-3
Number of pages: 41	**Number of attachments:** 9 annexes

Keywords:
Fish disease, survival, IHN, VHS, SVC, EHN, iridovirus, ISA, VER, VEN, nodavirus, IPN, BKD, *Renibacterium salmoninarum*, RTFS, *Flavobacterium psychrophilum*, SRS, *Piscirickettsia salmonis*, disinfection, inactivation, iodophor, ozone.

English summary:
This report constitutes a deliverable of the RTD project ¨Appraisal of the soo-sanitary risks associated with trade and transfer of fish eggs and sperm (Acronym: FishEggTrade)¨. It contains a review of scientific papers and other information pertaining to the survival of fish pathogens outside the host, and their susceptibility to disinfection procedures. The following fish diseases and their causative agents are addressed: Infectious hematopoietic necrosis (IHN), viral haermorrhagic septicaemia (VHS), spring viraemia of carp (SVC), epizootic haematopoietic necrosis (EHN) and other iridovirus infections, infectious salmon anaemia (ISA), nodavirus infections, infectious pancreatic necrosis (IPN), bacterial kidney disease (BKD), rainbow trout fry syndrome (RTFS), piscirickettsiosis. The report also comprises an overview of disinfection procedures commonly used in various countries.

Norsk sammendrag:
Rapporten utgjør en milepæl innen forskningsprosjektet ¨Appraisal of the soo-sanitary risks associated with trade and transfer of fish eggs and sperm (Akronym: FishEggTrade)¨. Rapporten inneholder en oversikt og vurdering av vitenskapelig litteratur og annen faglig informasjon vedrørende fiskesykdomsorganismers overlevelse utenfor verten, og deres følsomhet for vanlige desinfeksjonsmetoder. Følgende fiskesykdommer og deres etiogiske agens behandles: Infeksiøs hematopoetisk nekrose (IHN), hermorrhagisk virusseptikemi (VHS), Rhabdovirus carpio infeksjon, epizootisk hematopoetisk nekrose (EHN) og andre iridovirus-infeksjoner, infeksiøs lakseanemi (ILA), nodavirusinfeksjoner, infeksiøs pankreasnekrose (IPN), bakteriell nyresyke (BKD), *Flavobacterium psychrophilum* infeksjoner, piscirickettsiose. Rapporten inneholder også en oversikt over vanlige desinfeksjonsmetoder for rogn som brukes i ulike land.

Published by: VESO, PO Box 8109 Dep., N-0032 Oslo, Norway
Phone: +47 22961100 Fax: +47 2296 1101

Table of contents

Introduction

National and international trade in fertilised eggs and gametes for finfish aquaculture is in most parts of the world subject to strict zoo-sanitary regulations and health certification requirements, many of which are built upon rather old and partly scarce scientific data. Aim of this concerted action project is thus to scrutinise and re-assess the scientific basis for current zoo-sanitary control requirements. In the previously accomplished part of the project (Work Package 1), we have found that there is reasonable evidence for so-called "true" vertical transmission (infection of the developing embryo or transmission inside the fertilised egg) only for a limited number of finfish diseases. These are bacterial kidney disease (BKD), infectious pancreatic necrosis (IPN), salmon rickettsial syndrome caused by *Piscirickettsia salmonis*, and *Flavobacterium psychrophilium infections*. For a number of other infections, there are indications that vertical transmission may occur. In our opinion, this is more likely to be a contamination of the egg surface ("egg-associated transmission"). Infectious haematopoietic necrosis (IHN) and nodavirus infections of marine species (VER/VNN) may serve as examples of this category. The aim of the current work package is therefore to scrutinise the ability of the selected infectious agents to survive in the environment or on the egg surface, as well as their susceptibility to various disinfection procedures. The information here will thus serve as the scientific basis for justifying control measures in order to prevent or manage risks of vertical transmission of fish diseases.

Material and Methods

Based upon the background knowledge of the participants and literature databases, draft chapters (one of which was based upon input from an external expert) were produced and submitted to the co-ordinator, who distributed the materials as a working draft. During the fourth plenary project meeting, this draft and its interpretation of the scientific data was discussed in-depth among the partners and invited scientists external to the consortium. Following these discussions, the updated draft was distributed among the meeting participants for further improvement and scientific revision, which was co-ordinated by the National Veterinary Institute of Norway, who is the responsible partner for this work package.

Results

Rhabdovirus infections
Infectious haematopoietic necrosis (IHN)

A summary of the information obtained from work package 1, "Hazard identification for vertical transfer of fish disease agents", indicated that infectious haematopoietic necrosis (IHN) outbreaks had occurred in areas previously thought to be IHN-free following shipments of salmonid eggs from areas where the disease was known to occur. Additionally, in a small number of cases, progeny that originated from eggs from IHN virus-positive parents that had been iodophor disinfected prior to incubation and rearing in virus-free water were found to be infected, indicating the possibility for true vertical transmission of the virus. Infectious haematopoietic necrosis virus (IHNV) can be detected in milt and reproductive fluids, and was found to adsorb to sperm. However, there is evidence for significant antiviral activity in yolk components prior to the eyed-egg stage. Although more than a billion eggs have been imported into Chile, no outbreaks of IHN or VHS have been reported, suggesting that both viruses are not or are rarely transmitted vertically when eggs are disinfected in iodine. Additionally, no evidence for vertical transmission was obtained in experiments when examining progeny from IHNV infected parents or when eggs that originated from infected adults were tested for virus. In conclusion, there is some evidence that the virus may be truly vertically transmitted, but if at all possible, this appears to be a very rare and infrequent event.

Virus survival outside the host

A review of the literature on IHNV indicates that some of the information regarding IHNV lability to pH, cooling, freezing, heating, and the agent's capability to survive free in the environment is available but it is limited and occasionally contradictory.

Effect of pH

Briefly, virus infectivity was shown to be significantly reduced at pH 5 and 9 (Pietsch et al. 1977).

Effect of temperature

Previous studies evaluating the effect of temperature on the survival of IHNV have shown that at 4°C a loss of 99.9% infectivity of IHNV

required more than 20 weeks (Pietsch et al. 1977). Infectivity of virus could also be extended several years at -20°C in tissue culture media. (Pietsch et al. 1977). Gosting and Gould (1981) found that inactivation between 8°C and 38°C was initially rapid until 99.9% of the infectivity was lost, but thereafter the rate was slower. The virus may survive several weeks at 15°C but at 32°C complete inactivation occurs within hours. Generally, IHNV was shown to survive several weeks at 15°C but at 32 °C complete inactivation occurred within hours. At higher temperatures survival time is shorter (Pietsch et al. 1977; Gosting and Gould 1981).

The results of a recent set of studies conducted by LaPatra et al. (2001a) indicated that three isolates of IHNV obtained from rainbow trout from different years and that exhibited antigenic differences were stable at 25°C for 2 h and very susceptible at 56°C exhibiting a titre reduction of >99.99% within 30 min. However, at 37°C isolates from 1990 (184-90 and 220-90) decreased in titre after 30 min by >99.9% and 90.0%, respectively after 30 min. The isolate from 1982 (039-82) only exhibited a 50% titre reduction after 90 min. This suggests there may be thermal inactivation differences of IHNV isolates collected from different years, geographic locations, and potentially host species.

The effect of freezing

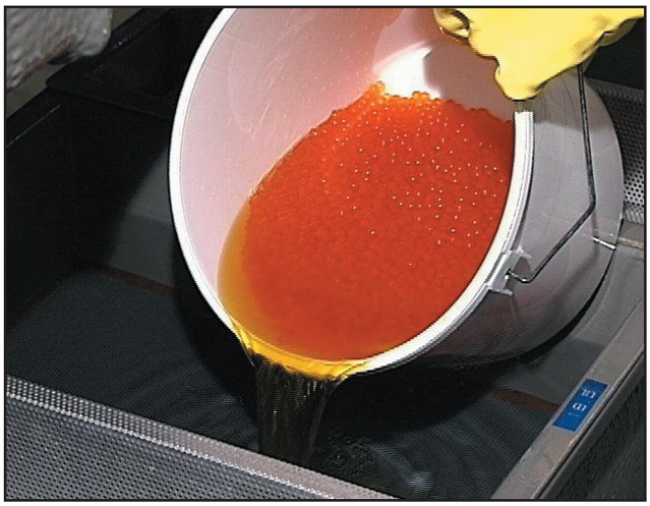
Previous studies regarding the effect of freezing had shown that IHNV infectivity could be extended several years at -20°C in tissue culture media, and virus preparations were shown to undergo several freeze thaw cycles with little effect on infectivity for cell cultures when high concentrations of dissolved protein are present. The medium and presence of protein was suggested to have significant effect on virus survivability (Pietsch et al. 1977). However, Watson et al. (1954) reported that a single freeze-thaw cycle reduced the virus titre by four orders of magnitude (from 10^6 to 10^2) and it was suggested that in fish products the effect of freezing is likely to be significant.

LaPatra et al (2001a) used twelve adult rainbow trout that died after injection of IHNV to further evaluate the effect of a freeze-thaw cycle on reducing virus titres in fish products. Approximately 75% of the fish that died, had detectable virus in either brain or kidney tissue when tested prior to freezing. The mean titre detected in brain tissue was 104.26 PFU/g (range, 102.00 to 105.70) and the mean titre in kidney tissue was 106.83 PFU/g (range, 104.00 to 107.30). No significant changes occurred in the concentration of IHNV in brain or kidney tissue after freezing at -20°C for 7 or 14 d. These results suggest IHNV is very stable, at least for short periods, through freezing and thawing in contrast to Watson et al. (1954) results. Similar results were obtained for another rhabdoviral pathogen of rainbow trout, viral hemorrhagic septicaemia virus (VHSV), further substantiating the negligible effect of a single freeze-thaw cycle on virus infectivity (Niels Jørgen Olesen, Danish Veterinary Laboratory, Aarhus, Denmark, personal communication). Although IHNV appears to be very stable through a freeze-thaw cycle there is no evidence of virus associated with processed rainbow trout from an area endemic for IHNV (LaPatra et al. 2001b).

Virus survival in different aquatic environments

In previous studies that assessed virus survival in different aquatic environments, IHNV was shown to survive in soft or hard lake waters at 10°C for 7 weeks at 10°C but for only 2 weeks in distilled water at the same temperature (Wedemeyer et al. 1978). In fresh water from fish hatcheries, dechlorinated city water, and double distilled water, IHNV survival time decreased with increasing temperature (Yoshimizu et al. 1986). Barja et al. (1983) showed that salinity was detrimental to IHNV survival. When survival of IHNV between fresh and marine waters was compared at 15 and 20°C, Toranzo and Hetrick (1982) found optimal survival in fresh water at 15°C. However, Kamei et al. (1987) found significant reduction in IHNV concentrations within 3 days at 15°C in untreated brackish and sea water and in estuary waters which contained sediments. Toranzo and Hetrick (1982) observed that viral inactivation occurred when high numbers of bacteria were present in the water. Kimura and co-workers identified a natural antiviral substance from fresh and marine waters obtained from different sources, including fish hatcheries, associated with the microflora (Yoshimizu et al. 1986; Kamei et al. 1988a, b).

When IHNV was seeded into spring water and incubated at 15°C, the virus appeared very stable in agreement with previous studies. However, for virus incubated in water obtained from a fish farm or river water, the virus titre decreased rapidly. Virus suspended in river water exhibited a 99% reduction in virus concentrations in 24 hours (LaPatra et al 2001a). This result is again consistent with previous observations and suggests for risk analysis purposes that studies be defined to mimic the situation being assessed as closely as possible so that false assumptions are not made.



Infectivity studies have indicated that the agent is able to survive for some time in the environment and that horizontal transmission may occur via faeces, urine and mucus in both freshwater and seawater (Mulcahy et al. 1983; LaPatra et al. 1989; Traxler et al. 1993). Demonstration of transmission via feed also indicates ability to survive outside the host (Wolf 1988).

Disinfection

Controlling the spread of IHNV by disinfection of hatchery water supplies has had some limited success. Methods such as ozonation, ultraviolet light, chlorination-dechlorination, and the addition of elemental iodine or other germicides such as ethanol, phenol and cresol soap solution, or methanol have all been shown to be effective in laboratory settings (Batts et al. 1991; Winton 1991; Inouye et al. 1992). In field trials, however, problems have arisen because of mechanical and electrical failures that occur routinely with equipment that must deliver a compound at a virus killing dose that is non-toxic to the fish. Nevertheless, the approach has sufficient promise that aquaculture facilities with limited or no SPF water are beginning to incorporate water disinfection equipment with some success.

Present control of IHNV relies upon hatchery practices that avoid the presence of the virus at the rearing facility. Eggs and fry are taken from stocks with no previous history of the virus and reared in specific pathogen free (SPF) water. When egg supplies from IHNV-negative fish are limited, the eggs from fish stocks having a history of IHNV outbreaks are treated with iodine-containing surfactant to inactivate virus on the eggs' outer surface (Amend and Pietsch 1972). Iodophor (100 mg/L) egg disinfection destroys at least 99.98% of IHNV on the surface of green and eyed eggs (Goldes and Mead 1995). In most cases this treatment has been successful in preventing the transmission of IHNV to progeny. Occasional outbreaks of disease have been observed in the progeny after treatment with iodophor and reared in SPF water. To some, this has suggested that vertical transmission may occur (Meyers 1990). However, if true vertical transmission does occur it most likely is a very infrequent event. There are several reports where IHNV-infected parents did not produce IHNV-infected progeny when the eggs and fish were incubated and raised in virus-free water and/or disinfected with an iodophor solution (Amend 1975; LaPatra 1990; Engelking et al. 1991; LaPatra et al., 1991;Yamazaki and Motonishi 1992; Traxler et al., 1997). Disinfection of freshly fertilized (green) and embryonated (eyed) salmonid fish eggs with organic iodine compounds (iodophors) is now recognized as a prudent fish culture technique in artificial production operations especially where viral pathogens such as IHNV are detected.

Historically malachite green has been used effectively to combat fungus infections of adult salmon and their eggs. In compliance with FDA regulations, a substitute chemotherapeutant, formalin, has been used more or less effectively. A potential benefit of using formalin instead of malachite green is its virucidal activity against enveloped viruses, such as IHNV. In-vitro comparison of IHN virus inactivation by these two compounds, at concentrations commonly used to treat adult salmonids, showed that formalin was more effective than malachite green against IHNV. Furthermore, the higher concentrations of formalin used to treat eggs exhibited an even greater potential for virus disinfection (LaPatra and Rohovec 1987). Although these test systems did not account for organic matter which could decrease the virucidal capacity of formalin, they did show a potential of limiting horizontal or fish-to-fish transmission of IHNV between adults and of further external virus disinfection of eggs during incubation

Priority research needs regarding pathogen survival and disinfection

1) Develop standardized methods to assess the stability of IHNV in different media and under different physical and chemical environments.

2) Assess the susceptibility of IHNV to different physical and chemical parameters under more "natural" conditions and develop quantitative data on the rate of inactivation of IHNV.

3) Develop additional information on how to reproduce an IHNV "life cycle" and how to accurately determine whether IHNV is in fact inactive.

Viral haemorrhagic septicaemia (VHS)

A summary of the information obtained from work package 1, "Hazard identification for vertical transfer of fish disease agents", indicated no evidence of true vertical transmission of viral haemorrhagic septicaemia (VHS) virus. This is in contrast to IHNV where few reports suspected cases of true vertical transmission. For many years eggs were exported from Denmark to UK also from VHS infected farms without any outbreak of the disease in UK. In addition, more than a billion of salmon and rainbow trout eggs have been imported into Chile, and no outbreaks of VHS have been reported, suggesting that VHSV is not or extremely rarely transmitted vertically when the eggs are iodine disinfected. In conclusion, there is no evidence that the virus is truly vertically transmitted. It has been experienced several times that the introduction of non-disinfected green eggs from infected farms in the incubation phase of a VHS infection will obviously result in a VHS outbreak (H. Korsholm, Danish Veterinary Services, personal communication).

IHNV and VHSV are closely related viruses belonging to the same genus and both affect salmonids at low water temperature condi-

Table 1. Overview of studies on inactivation of infectious haematopoietic necrosis virus (IHNV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	28 °C	MEM-1	330 min	90 %	Gosting and Gould 1981
	32 °C	MEM-1	90 min	99.9 %	Gosting and Gould 1981
	38 °C	MEM-1	15 min	99.9 %	Gosting and Gould 1981
	35 °C	MEM-0	5 hours	inactivated	Whipple and Rohovec 1994
	40 °C	MEM-0	20 min	inactivated	Whipple and Rohovec 1994
	45 °C	MEM-0	10 min	inactivated	Whipple and Rohovec 1994
	50 °C	MEM-0	90 sec	inactivated	Whipple and Rohovec 1994
	55 °C	MEM-0	30 sec	inactivated	Whipple and Rohovec 1994
UV-C	10–30 J/m^2	Strain dependent		99 %	Yoshimizu et al. 1986
	20 J/m^2			99.9 %	Sako and Sorimachi 1985
Acid	pH 4 Citric phos. buffer	22 °C,	7 hours	incomplete	Whipple and Rohovec 1994
	pH 3.8-4.3 fish silage	22 °C,	30 sec	inactivated	Whipple and Rohovec 1994
NaOH					
Chlorine	0.1 mg/l	10 °C, aqua dest.	30 sec	inactivated	Wedemeyer et al. 1978
	0.5 mg/l	10 °C, soft lake water	5 min	inactivated	Wedemeyer et al. 1978
	0.5 mg/l	10 °C, hard lake water	10 min	inactivated	Wedemeyer et al. 1978
	1 mg/l	10 °C, hard lake water	30 sec	inactivated	Wedemeyer et al. 1978
	10 ppm		30 min	inactivated	Amend and Pietsch 1972
Formalin	0.2 %		60 min	incomplete	Amend and Pietsch 1972
Ozone	0.01 mg/l	10 °C, aqua dest.	30 sec	inactivated	Wedemeyer et al. 1978
	70 mg/h/l	10 °C, soft and hard lake water	10 min	inactivated	Wedemeyer et al. 1978
Iodophor	25 ppm	pH 7.0	15 sec	inactivated	Amend and Pietsch 1972
	12 ppm	pH 7.0	30 sec	inactivated	Amend and Pietsch 1972

tions. Therefore it can be presumed that studies on virus stability on one of the viruses also will be true for the other.

Virus survival outside the host

VHSV was reported to survive 49 days at 10°C in tap water. When suspended in mud VHSV was stable for 10 days at 4°C (Ahne, 1982a). Drying the virus for 28 days at 4°C and 20°C, respectively only reduced virus titre with 99% (2 log) according to Ahne 1982b. According to Jørgensen 1973, however, total inactivation (> 7 log) was obtained within 3 weeks. VHSV survive freeze drying and freezing at -20°C for years (N. J. Olesen, pers. obs.). It was reported that the time required to produce a 3 log10 inactivation of VHSV was several years at -20°C, several months at 4°C, approximately 4 weeks at 20°C, and less than one min at 70°C. It was reported that in a dry environment, the virus survived for approximately one week at 4°C (Frost and Wellhausen, 1974; Pietsch et al., 1977). VHSV is completely inactivated within hours at temperatures between 35 and 50 °C and within minutes at higher temperatures.

Addition of foetal calf serum (FCS) stabilizes the infectivity at elevated temperatures significantly compared with virus in serum free medium (Frost and Wellhausen, 1974). VHSV is thus rather

unstable outside the host and spreading of the virus is possible to avoid if simple and basic preventative measures are followed. The most common route of infection is downstream by water flow, upstream by migrating fish and cross water catchments by fish predating birds (herons, cormorants, crows and gulls) and not to forget human activities and trade.

Survival of Japanese VHSV isolates in untreated sea water was short, few days at 15° and up to 25 days at 4°. When the sea water was treated by autoclavation or 0.22 µm filtered virus survival was prolonged significantly (60 d at 15°C and 32 d at 20°C) which, however, still was less than survival in freshwater (measured to 40 days at 25°C and >60 days at lower temperatures) (Mori et al. 2002). It may be concluded that it is primarily the numbers of bacteria and virus inhibiting compounds in the water and not the salinity that accounts for virus inactivation.

Disinfection
Being a membrane virus VHSV is susceptible to many disinfectants, except halogens as chlorine, where exposure up to 540 mg/l for 2-20 min was needed (Ahne 1982 b).

Chemicals regularly used for water treatment in connection with parasite and fungus infections as methylene blue, malachite green, benzalkonium chloride, and copper sulphate have according to Ahne (1982a, 1982b) no effect on VHSV. Kurita et al. (2002), however, demonstrated that the minimum effective concentration of a 10% benzalkonium chloride product for VHSV disinfection was 1:1000 at 30 min exposure Methanol and ethanol had no effect on VHSV, whereas propanol was effective disinfecting VHSV within 30 sec in a 30% concentration (Kurita et al. 2002). Formalin 2 - 3% of 40% formaldehyde solution inactivates the virus within 5 min (Jørgensen 1973, Ahne, 1982b).

Iodophors as Actomar K30 which is widely used for egg disinfection inactivate the virus within 4 min at 100 ppm under both clean and dirty conditions. At 50 ppm some rest infectivity was observed after 30 min under dirty conditions, but not under clean conditions (Ahne and Held 1980).

The UVC irradiation doses necessary for 99.9% inactivation of VHSV is only 7.9 J m-2 , compared to 1188 J m-2 for IPNV (Øye and Rimstad, 2001).

pH
VHSV is susceptible to both low and high pH. pH 3 will reduce infectivity within 3 hours while NaOH (pH 12) will inactivate the virus within 5-10 min (Ahne 1982 b). VHSV was reported to have survived for 10 minutes at pH 2.5 and 2 hours at pH 12.2 (Jørgensen, 1974).

Salinity
Studies by Kurita et al. (2002) revealed that VHSV of marine origin (Japanese flounder) was equally susceptible under fresh water and sea water conditions towards disinfectants as iodophor (50 ppm, 1 min) whereas the effect of chlorine apparently decreased under artificial seawater condition.

The table given in the most recent version of the OIE manual on aquatic animal health on disinfection procedures should be followed and will cover most of the demands for VHSV: ¨http://www.oie.int/eng/normes/fmanual/A_00014.htm¨

Priority research needs regarding pathogen survival and disinfection

1. Test efficiency of gentle long-term heat treatment as an easy and low cost disinfection method of equipment.

2. Test systematically modern and more environmentally desirable and commercially available disinfectants.

3. Test virus survival by treatment with chemicals used for reduction of parasite burden.

4. Test if constant UV/ozone treatment of inlet water in recirculated aquaculture facilities will be able to prevent the introduction of fish pathogenic rhabdoviruses.

Spring viraemia of carp (SVC)
A summary of the information obtained from work package 1, ¨Hazard identification for vertical transfer of fish disease agents¨, indicated no evidence of true vertical transmission of spring viraemia of carp virus (SVCV, synonym: *Rhabdovirus carpio*, RVC). This is in contrast to IHNV where few reports suspected cases of true vertical transmission. Very few studies on the stability of SVC virus have been performed and therefore the related rhabdoviruses IHNV and VHSV are used as references. Carps are usually hatched on the site of spawning due to very short incubation and the fragility of eggs, therefore trade of carp egg and sperm is not very common compared to the trade of salmonid eggs.

Virus survival outside the host
In sterile cell culture medium, SVC virus survived for less than two weeks at 23°C and less than half a year at 4°C. The survival was several times longer when foetal calf serum (FCS) was added to the medium, and was increased at lower temperatures (Ahne, 1976). SVCV was reported to survive 42 days at 10°C in tap water. When suspended in mud, SVCV was stable for 42 days at 4°C as well (Ahne, 1982b). Drying the virus for 28 days at 4°C and 20°C, respectively only reduced virus titre by 2 - 3 log (Ahne 1982b) and in presence of FCS, SVCV survived multiple freeze-thaw cycles, freeze

Table 2. Overview of studies on inactivation of viral haemorrhagic septicaemia virus (VHSV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	30 °C		24 hrs	99.9 %	Vestergård-Jørgensen 1974
	50 °C		10 min	99.9 %	Vestergård-Jørgensen 1974
	70 °C		1 min	99.9 %	Vestergård-Jørgensen 1974
UV-C	7,9 Jm^{-2}	freshwater		99.9 %	Øye and Rimstad 2001
Acid	pH 2.5		10 min	99.9 %	Vestergård-Jørgensen 1974
	pH 3.0		60 min	99.9 %	Ahne 1982 b
	pH 3.0		180 min	inactivated	Ahne 1982 b
NaOH	pH 12	2 %	5-10 min	inactivated	Ahne 1982 b
	pH 12.2		2 hours	99.9 %	Vestergård-Jørgensen 1974
Chlorine	7.60 mg/l	10 °C	10 min	99 %	Ahne 1982 b
	25-54 mg/l	10 °C	5 min	99 %	Ahne 1982 b
	98 mg/l	10 °C	2 min	99 %	Ahne 1982 b
	25-98 mg/l	10 °C, 2.5 % FCS	10 min	99 %	Ahne 1982 b
	515 mg/l	10 °C, 2.5 %FCS	2 min	99 %	Ahne 1982 b
Formalin	2 %		5 min	99.9 %	Jørgensen 1973
	3 %	10 % FCS	5 min	inactivated	Ahne 1982 b
Methanol	40 %	in PBS, 15ºC	300 sec	ineffective	Kurita 2002
	40 %	in SW, 15ºC	300 sec	ineffective	Kurita 2002
Ethanol	40 %	in PBS, 15ºC	300 sec	ineffective	Kurita 2002
	40 %	in artificial SW, 15ºC	120 sec	inactivated	Kurita 2002
Propanol	30 %	in PBS, 15ºC	30 sec	inactivated	Kurita 2002
	20 %	in artificial SW, 15ºC	120 sec	inactivated	Kurita 2002
Phenol	2.5 %	in PBS, 15ºC	5 min	inactivated	Kurita 2002
	2.5 %	in artificial SW, 15ºC	5 min	inactivated	Kurita 2002
Cresol	0.1 %	in PBS, 15ºC	5 min	inactivated	Kurita 2002
	0.25 %	in artificial SW, 15ºC	15 min	inactivated	Kurita 2002
Quarternary ammonium	1 %		6 hrs	ineffective	Ahne 1982 b
	1:1000 dilution	in PBS, 15ºC	30 min	effective	Kurita 2002
	1:1000 dilution	in artificial SW, 15ºC	5 min	effective	Kurita 2002
Iodophor	100 ppm		4 min	inactivated	Ahne and Held 1980
	50 ppm	in PBS, 15ºC	1 min	effective	Kurita 2002
	50 ppm	in artificial SW, 15ºC	1 min	effective	Kurita 2002
Sodium hypochlorite	50 ppm	in PBS, 15ºC	1 min	effective	Kurita 2002
	100 ppm	in artificial SW, 15ºC	5 min	effective	Kurita 2002
Bleaching powder	50 ppm	in PBS, 15ºC	2.5 min	effective	Kurita 2002
	50 ppm	in artificial SW, 15ºC	60 min	effective	Kurita 2002

drying and freezing at -20°C for years (de Kinkelin and Le Berre, 1974; B.J. Hill, pers. obs.). SVCV is completely inactivated within few days or hours at temperatures between 35 and 50 °C and within minutes at higher temperatures (Ahne, 1976).

SVCV is thus rather unstable outside the host and spreading of the virus is possible to avoid if simple and basic preventative measures are followed. The most common route of infection is downstream by water flow, upstream by migrating fish and cross water catchments by fish predating birds (herons, cormorants, crows and gulls) and not to forget human activities and trade.

Disinfection

Being a membrane virus SVCV is susceptible to many disinfectants, except halogens as chlorine, where exposure up to 540 mg/l for 2-20 min was needed (Ahne 1982b). Chemicals regularly used for water treatment in connection with parasite and fungus infections as methylene blue (20 mg/l), malachite green (10 mg/l), benzalkonium chloride (1%), and copper sulphate (100 mg/l) have no effect on SVCV.

3 % formalin (3% by volume of a 40% formaldehyde solution) inactivates the virus within 5 minutes (Ahne 1982a, 1982b). Iodophors as Actomar K30, which is widely used for egg disinfection, inactivate the virus within 4 min at 100 ppm under both clean and dirty conditions. At 50 ppm some rest infectivity was observed after 30 min under dirty conditions but not under clean conditions (Ahne and Held 1980).

pH

SVCV is susceptible to both low and high pH. pH 3 will reduce infectivity within 3 hours while NaOH (pH 12) will inactivate the virus within 5-10 min (de Kinkelin and Le Berre, 1974; Ahne 1982b).

Table 3. Overview of studies on inactivation of spring viraemia of carp virus (SVCV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	30ºC		480 min	50 %	Ahne 1976
	35ºC		480 min	90 %	Ahne 1976
	40ºC		120 min	98 %	Ahne 1976
	45ºC		20 min	98 %	Ahne 1976
	50ºC		5 min	98 %	Ahne 1976
pH	pH 11		6 days	ineffective	Kölbl 1975
	pH 12	2 % NaOH	5-10 min	inactivated	Ahne 1982
	pH 6.1	2 % serum	6 days	ineffective	Kölbl 1975
	pH 3		120 min	99 %	Ahne 1976
Chlorine	7.60 mg/l	10 °C	20 min	99 %	Ahne 1982
	27 mg/l	10 °C	10 min	99 %	Ahne 1982
	36-55 mg/l	10 °C	2 min	99 %	Ahne 1982
	101 mg/l	10 °C, 2,5 % FCS	2 min	99 %	Ahne 1982
	506 mg/l	10 °C, 2,5 %FCS	2 min	99 %	Ahne 1982
	3 %	10 % FCS	5 min	inactivated	Ahne 1982
Formalin	1 %		6 hrs	ineffective	Ahne 1982
Quaternary ammonium	100 ppm		4 min	inactivated	Ahne and Held 1980
Iodophor					

The table given in the most recent version of the OIE Manual of diagnostic tests for Aquatic Animals on disinfection procedures should be followed and will cover most of the demands for SVCV: http://www.oie.int/eng/normes/fmanual/A_00014.htm

Priority research needs regarding disinfection and survival

Priority should be given to basic studies of SVC virus survival in water under conditions relevant for potential disease transmission in European aquaculture.

Epizootic haematopoietic necrosis (EHN)

Epizootic haematopoietic necrosis (EHN)

In WP1 it was reported that no publications or other reports dealing with the issue of possible vertical transmission of EHN or on the detection of the causative virus(es) in gonads, sperm, ovarian fluid or eggs of susceptible host species have been found despite extensive searches of scientific literature databases and communications with external experts working on these viruses. In a personal communication, the designated expert at an OIE Reference Laboratory for EHN, states that there are no records of EHNV having been recovered from gonads, sperm, ovarian fluid or eggs of affected European perch or rainbow trout populations in Australia. Furthermore, there are no published field observations or any other indirect evidence to suggest that vertical transmission of EHNV may occur. In the absence of any available evidence either for or against vertical transmission of EHN or the presence of the causative virus(es) in the sexual products of susceptible host species, the likelihood of vertical transmission of infection is impossible to assess at the present time.

Also in the report for WP1, it was stated that further research on characterisation of the virus is needed to resolve the uncertainty as to whether ESV and/or ECV are true strains of EHNV or different viruses. In a subsequent personal communication, Dr Alex Hyatt, the designated expert at an OIE Reference Laboratory for EHN, has confirmed that this work has been done and it is now accepted by the International Committee for Taxonomy of Viruses (ICTV) that ESV and ECV are different strains of the same virus. However, this virus is not the same species as EHNV. Therefore, ESV and ECV will not be considered further for the purposes of this project.

Virus survival outside the host

Only a limited amount of data is available on the viability of EHNV outside the host and on its susceptibility to disinfection. In the only published study, Langdon (1989) found that cell-free EHNV suspensions in distilled water showed no decrease in infectivity titre over 97 days and in infected cell cultures held at 4°C the virus retained infectivity even after 300 days (table 4). In dried infected tissue culture medium the infectivity was still present after 113 days at 15°C but had been lost completely by 200 days, and infectivity was still present in infected tissue culture medium and fish tissues stored frozen at -20 or -70°C after 2 years. All infectivity was also lost when infected tissue culture medium was held at 60°C for 15 minutes or at 40°C for 24 hours.

Disinfection

All infectivity was inactivated within 2 hours when dried infected tissue culture medium was overlaid with 70% ethanol but some infectivity remained after 5 hours following similar treatment with 400 ppm sodium hypochlorite. Virus in tissue culture medium treated by adjusting the pH to 4.0 or 12.0 lost all infectivity within 1 hour and within 2 hours when treated by addition of 200 ppm sodium hypochlorite.

These findings led the author to conclude that disinfection of fish farm equipment would be best achieved by cleaning any dried surface films then treating them with 200 ppm sodium hypochlorite. Application of lime to achieve high pH would probably be effective for decontaminating earthen ponds. However, the persistence of EHNV infectivity in pond sediments or in natural waters was not determined.

There have been no studies reporting on the efficacy of iodophor disinfectants against EHNV or of the effect of UV or ozone treatment on the infectivity of EHNV in contaminated water. Neither is there any information of the toxicity of iodophors to eggs of European perch at the time of or after fertilisation.

Table 4. Overview of studies on inactivation of epizootic haematopoietic necrosis virus (EHNV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	40 °C		24 hrs	inactivated	Langdon 1989
	60 °C		15 min	inactivated	Langdon 1989
Acid	pH 4		1 hr	inactivated	Langdon 1989
NaOH	pH 12		1 hr	inactivated	Langdon 1989
Ethanol	70 %	Dried tissue culture medium	2 hrs	inactivated	Langdon 1989
Chlorine	200 ppm	Tissue culture medium	2 hrs	inactivated	Langdon 1989
	400 ppm	Dried tissue culture medium	5 hrs	incomplete	Langdon 1989

Priority research needs regarding disinfection and survival

The first research priority, as recommended in the report of WP1, is to perform available testing procedures for EHN virus on gonad, milt or ovarian fluids of breeding perch or rainbow trout in infected populations in Australia. If these tests demonstrate the presence of infectious EHNV and the possibility of vertical transmission, studies on the efficacy and toxicity of iodophor disinfection along the lines of the studies on vertical transmission of IHNV and IPNV trout eggs should be carried out.

Studies need to be conducted to determine the dosages of UV or ozone required to inactivate infectivity of EHNV in contaminated water supplies or effluents in trout hatcheries.

Infectous salmon anaemia (ISA)

Infectious salmon anaemia (ISA)

There is no hard evidence for the vertical transmission of ISAV neither as an egg-associated contamination nor as true vertical transmission inside the egg, and vertical transmission is obviously insignificant in the epidemiology of the infection. Whereas external contamination of gametes or embryos cannot be excluded, the true vertical transmission of ISAV appears unlikely as deemed from the information currently available. Any effective disinfectant that is able to inactivate the ISA virus on the surface of eggs without harming the eggs, will be acceptable from a trade point of view as necessary measure to prevent spread of ISA by eggs.

Virus survival outside the host

There are few published studies regarding the stability of ISAV outside the host. Falk et al. (1997) showed that ISAV were stable in the pH range 5 – 9. At pH 4 the virus was completely inactivated after 30 min, and at pH 11 a 90 % reduction in infectivity was observed after 30 min. The virus was stable for 14 days at 4 °C, and for 10 days at 15 °C. At 37 °C, a 99,9 % titre reduction was observed after 24 hours. Incubation at 56 °C completely inactivated the virus in 5 minutes. In transmission trials with infective tissue homogenate, Torgersen (1998) showed that all infectivity was lost after treatment at 50 °C for 2 minutes and 55 °C and 60 °C for 1 minute. In a similar study, exposing ISA infective material in sea water and fresh water at 10 °C, it was shown that infectivity was reduced after 24 – 48 hours, but some infectivity was still present after 48 hours. It was not possible to determine any difference in sea water contra fresh water stability. Rimstad (pers. comm. 2003) observed ISAV stability upon suspension in autoclaved full strength sea water (35 ‰) at 4 °C, and found a 99,9 % titre reduction after 4 months. Although not representative for natural conditions, this result indicates that ISAV could be able to survive for an extended period in sea water. Five cycles of freezing (-80 °C) and thawing (20 °C) did not reduce infectivity (Falk et al. 1997).

Disinfection

Only a few studies have been performed to assess the susceptibility of the ISA virus towards disinfection. It was not until 1995 that one was able to grow ISAV in cell culture, but some studies using transmission trials with infected tissue were performed prior to this. In preliminary tests using ISA infected tissue, Krogsrud et al. (1991) studied the efficiency of heat, sodium hydroxide, formic acid, chlorine, ozone, UV and formalin. In a follow up study to these trials, Torgersen (1998) examined the efficiency of several physical and chemical disinfection methods (heat, formic acid, UV, sodium hydroxide, chlorine) towards ISA infected tissue. The results from these experiments are summarized in the table below. Øye and Rimstad (2001) studied the UV inactivation of ISAV, IPNV and VHSV suspended in different water qualities, and found ISAV to be very susceptible to UV. In recent years, a few studies have been performed to determine the in vitro efficiency of several commercial disinfectants, like iodophors, chlorine-based disinfectants, peroxygen-based products etc. Smail et al. (2001) found iodophors, chloramine T and chlorine dioxide diluted in hard water at 4 °C to be effective after 5 min. contact time using the manufacturers recommended dose. In a comprehensive Canadian study (Anonymous 2002) several disinfectants were tested for their efficiency against ISAV. Iodophors, chlorine, peroxygen compounds and chlorhexidine inactivated ISAV after 15 min at 15 °C, but iodophors and chlorhexidine lost their efficiency in the presence of blood/mucus. As far as we know, no studies aiming directly at egg disinfection have been published as of yet.

Tables 5 gives an overview over inactivation experiments carried out over the years since ISA first was diagnosed, and of recommendations for disinfection.

Table 5: Overview of studies on inactivation of infectious salmon anaemia virus (ISAV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	45°C		5 min	still infective	Torgersen 1998
	50°C		1 min	still infective	Torgersen 1998
	50°C		2 min	inactivated	Torgersen 1998
	55°C		1 min	inactivated	Torgersen 1998
	55°C		10 min	inactivated	Krogsrud et al. 1991
UVC	5 J/m^2	diluted ISA-infective tissue homogenate		still infective	Torgersen 1998
	40-100 J/m^2				Torgersen 1998
	200 Jm^{-2}			inactivated	Torgersen 1998
	33 ± 3.5 J/m^2	FW		3-log reduction	Øye and Rimstad 2001
	51 ± 13 J/m^2	SW		3-log reduction	Øye and Rimstad 2001
	72 ± 16.31 J/m^2	wastewater		3-log reduction	Øye and Rimstad 2001
	1200 Jm^{-2}	processing plant effluents			Anonymous, Scottish Executive 2000
Formic acid	pH 3.5	0°C, H_2CO_2	8 hours	inactivated	Torgersen 1998
	pH < 3.9		24 hours		Anonymous 2000
	pH 4,0	0°C, H_2CO_2	24 hours	inactivated	Torgersen 1998
NaOH	pH 11,5	0°C	48 hours	inactivated	Torgersen 1998
	pH 12	0°C	24 hours	inactivated	Torgersen 1998
	pH 12		7 hours	inactivated	Krogsrud et al. 1991
	pH > 12		7 hours		Anonymous 2000
Chlorine	50 mg/l		30 min	still infective	Torgersen 1998
	100 mg/l		15 min	inactivated	
Formalin	0,5 %			inactivated	Krogsrud et al. 1991
	0,5%		16 hours		Anonymous 2000
Ozone	8 mg/l	~ 600-750 mV redox potential	4 minutes		Anonymous 2000
Virkon S (peroxygen compound)	1:100 and 1:200 in hard water	tested on ISAV in SHK cell culture	10 and 20 min at 20°C	virucidal activity confirmed	Antec 2003 AVC 2002

Different brands of iodophors have considerd for disinfection of prehardened- and eyed eggs (table 6). A 100 ppm iodophor solution has been recommended for the disinfection of eggs:

Table 6. Recommendations for nactivation of ISAV with iodophor-based compounds

Method	Dose	Comment	Contact time	Caution warning	Reference
Iodophor	100 mg/l	eyed eggs and pre-hardened eggs	5 minutes		anonymous, Scottish Executive 2000
	100-200mg/l	equipment etc.	5 minutes		
Wescodyne	144 mg/l	general use		blood contamination	Anonymous, 2001
GermKill	100 mg/l	general use		effective	
Ovadine	> 100 mg/l	general use		undetermined effect	
Buffodine	114 mg/l	egg bath	>30 minutes	mucus and/or blood contamination	

Priority research needs regarding pathogen survival and disinfection

1. Further studies, using standardized methods, on the stability of ISAV under different environmental conditions (fresh water, sea water) at various temperatures, and with/ without the presence of natural micriobial flora.

2. Repeat the studies of Krogsrud et al. (1991) and Torgersen (1998) using standardized methods in order to get quantitative data. Determine the efficiency of various disinfectants under natural conditions (cold water, organic loading, salinity, etc.).

3. For disinfection of eggs, further research into new compounds and disinfection regimes for inactivation of ISAV should be carried out. Furthermore, testing of eggs from infected broodfish under farming conditions should be carried out in order to gain experience from the field as regard the risk for transmission of ISA with un-disinfected eggs compared to disinfected eggs.

Viral encephalopathy and retinopathy/ Viral nervous necrosis (VER/VNN)

Since VER is usually detected in larvae and juveniles even in hatcheries supplied with UV treated water, vertical transmission has been suspected by several authors. In recent years the causal agent has often been detected by different methods in gonads of spawners of several species. Surprisingly, no virus isolation has been reported from gonads suggesting that infection is present at undetectable level with regard to the cell culture method.

Recently, the virus has been transmitted to the eggs and larvae obtained from spawners experimentally infected by IM injection.These results clearly suggest that infected parents may transmit infection to their offspring but it is still to be proven if the virus is inside or outside the eggs. This information is very important in order to establish the risk of VER/VNN spread by egg trade.

Actually, trade in marine fish eggs does not represent a real risk for disease spreading, mainly because these eggs, unlike salmonid eggs, usually are hatched at the production site. Nevertheless, during recent years, a growing market for one day old sea bass larvae from some European marine hatcheries to different countries in the West Indies/Arabic countries or other destinations has been established (A. Le Breton, pers. comm.). When broodstock are infected, the risk of VER/VNN virus spreading by sea bass larvae has to be considered. Furthermore, it must be kept in mind that viral agents may easily be spread at farm level from infected parents to offspring. For these reasons, egg disinfection should be compulsory applied as a basic preventive method in marine aquaculture as it is in freshwater aquaculture.

Pathogen survival outside the host

Total inactivation is obtained following a seven day period in dry conditions (Maltese and Bovo, 2001). High temperatures may affect the virus which is completely inactivated after four-day exposure at 37°C while it remains fully pathogenic during a six-month period at 15°C. Alkaline conditions (pH 12) are more harmful than acid conditions and the virus is inactivated in a short time at pH 12 (Arimoto et al., 1996). The virus is sensitive to UV exposure and the reported I.D.99 is 36,000 µWsec/cm2 (Maltese and Bovo, 2001).

Disinfection

Little information is available on the efficacy of egg disinfection in marine species and particular in disinfection against VER/VNN virus. Furthermore, the large number of species susceptible to VER/VEN does not allow the establishment of a definite protocol mainly due to the potential difference in toxicity of iodophors or other available substances to eggs of such species.

Only few papers have been published so far on the efficacy of available common disinfecting agents used in aquaculture. On a few occasions the reported data are not in complete agreement with data known for other agents. Table 7 summarizes reported results on disinfection.

Virus inactivation has been reported following benzalkonium chloride exposure (Arimoto et al., 1996). This observation needs to be confirmed because quaternary ammonium compounds have been reported to be effective only on enveloped agents. Low iodophor concentrations (25-100 ppm) show a strong effect on the pathogen (Arimoto et al. 1996; Frerichs et al. 2000 and Maltese and Bovo 2001). Similar results have been obtained with chlorine (Arimoto et al., 1996; Frerichs et al., 2000) but the presence of organic substances may significantly reduce their efficacy (Frerichs et al., 2000).

Data published concerning the use of formalin suggest a poor effect of this chemical on VER/VNN agent even when applied for several hours (Frerichs et al., 2000). Use of formalin for disinfection of contaminated surfaces should therefore not be recommended. Since formalin is known to be effective on several naked viruses like IPNV, this result should be re-confirmed by further investigations.

Ozone has been proposed for the disinfection of eggs in different marine species against VER/VNN virus contamination. In Norway halibut, turbot and cod eggs are routinely disinfected by ozone treatment (R. Johansen NVI Oslo and S. Grotmol Univ. of Bergen, pers. comm.). According to Grotmol and Totland (2000) ozone treatment of experimentally infected halibut eggs inactivates egg surface vi-

ral particles and may reduce the risk of transmission of nodavirus to hatching larvae. Nevertheless, the procedure used seems not completely effective (Johansen and Grotmol, 2003 pers. comm.). Similar uncertainty also exists as regards cod and turbot eggs. This last observation suggests the existence of a true VER/VNN vertical transmission in these species.

In Greece, sea bass egg disinfection is only occasionally applied by iodophors and the efficacy is unknown (Varvarigos 2003, pers. comm.). The same situation occurs in Spain where only some hatcheries disinfect the eggs mainly to reduce bacterial load on the egg surface (Padros, 2003 pers. com.). Eggs are exposed for 10 min in 50 ppm iodophors.

In Israel vertical transmission is suspected in: barramundi, different grouper species, red drum, grey mullet and sea bass but no egg disinfection strategies have been so far applied (Ucko, Diamant and Colorni, 2003 pers.comm.). In Italy the eggs coming from the most important hatcheries are exposed for 10 min at 50-100 ppm iodophors.

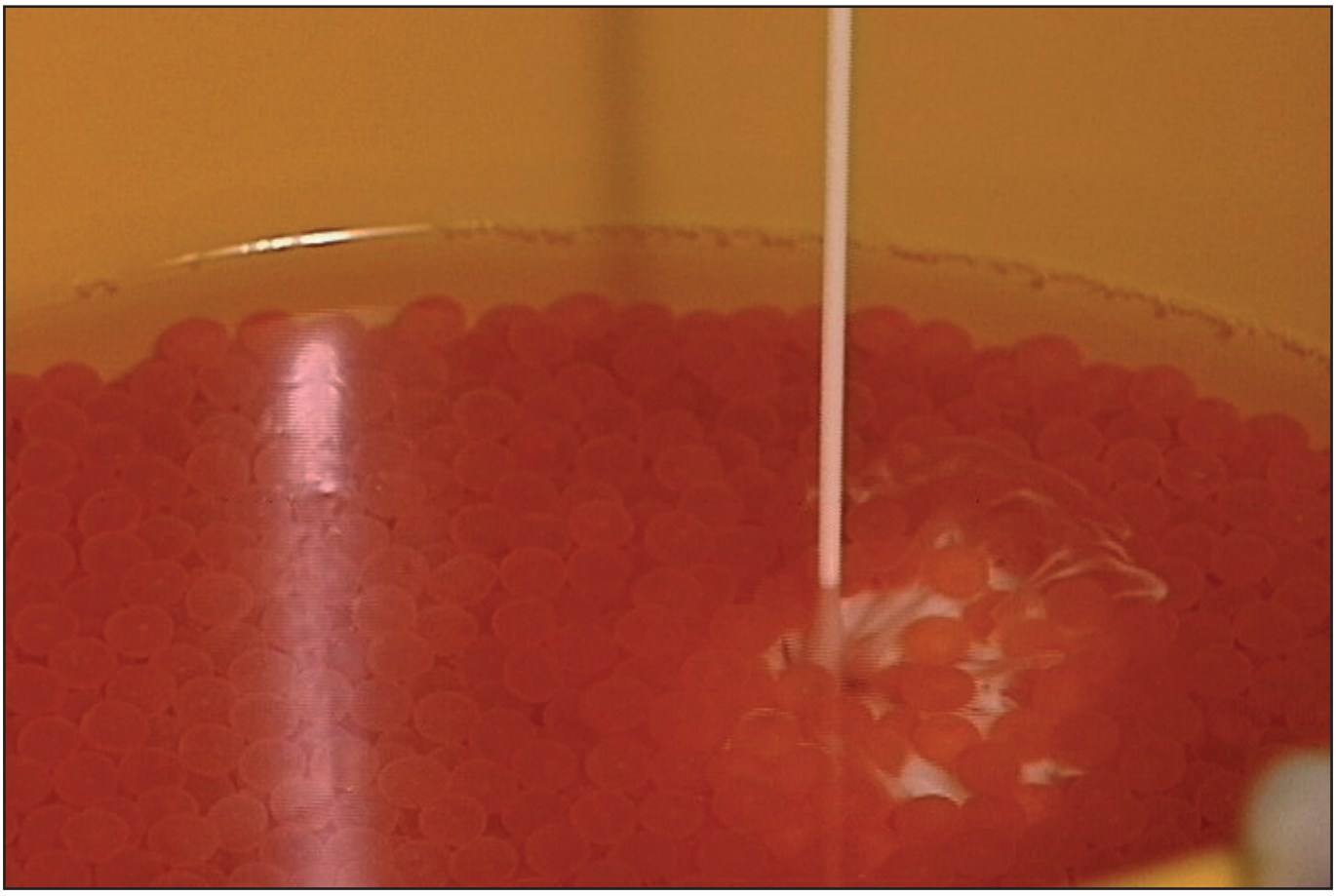


Table 7. Overview of studies on inactivation of nodaviruses (VER/VNN virus)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Drying	-	SBVERV-475-98-	7 days	inactivation	Maltese and Bovo 2001
Heat	15°C	SBNNV	6 months	no inactivation	Frerichs et al. 2000
	25°C	SBNNV	3 months	inactivation	Frerichs et al. 2000
	25°C	SBVERV-475-98	1 month	inactivation	Maltese and Bovo 2001
	29°C	SBVERV strain V26	1 month	inactivation	Peducasse et al., 1999
	37°C	SBNNV-	4 days	inactivation	Frerichs et al. 2000
	37°C	SBVERV strain V26	5 days	inactivation	Peducasse et al., 1999
	60°C	SBNNV-	60 min	inactivation	Frerichs et al. 2000
	60°C	SJNNV	30 min	inactivation	Arimoto et al.* 1996
	70°C	SBVERV-475-98	10 min	inactivation	Maltese and Bovo 2001
UV-C	2112 J/m^2	SBNNV-15°C		99,9%	Frerichs et al. 2000
	984 J/m^2	SJNNV		inactivation	Arimoto et al.* 1996
	1080 J/m^2	SBVERV-475-98 DI99=36000 µWsec/cm^2		99,9%	Maltese and Bovo 2001
Acid	pH 2	SBNNV-15°C	15 days	99%	Frerichs et al. 2000
	pH 2	SBVERV-475-98	15 days	90%	Maltese and Bovo 2001
	pH 2	SBNNV-15°C	42 days	inactivation	Frerichs et al. 2000
	pH 3	SJNNV- 20°C	10 min	no inactivation	Arimoto et al.* 1996
	pH 7	SJNNV- 20°C	10 min	no inactivation	Arimoto et al.* 1996
NaOH	pH 11	SBVERV strain V26	48 hours	inactivation	Peducasse et al.,1999
	pH 11	SBVERV-475-98	48 hrs	99%	Maltese and Bovo 2001
	pH 11	SBNNV-15°C	3 days	inactivation	Frerichs et al. 2000
	pH 11	SBVERV-475-98	5 days	inactivation	Maltese and Bovo 2001
	pH 11	SBNNV-15°C	15 days	inactivation	Frerichs et al. 2000
	pH 12	SJNNV-20°C	10min	inactivation	Arimoto et al.* 1996
	pH 14	SBVERV-475-98	60 min	inactivation	Maltese and Bovo 2001
Formalin	2%	SBNNV-15°C	6 hrs	99.99 %	Frerichs et al. 2000
	>1,6%	SJNNV	10 min	no inactivation	Arimoto et al.* 1996
Quarternary ammonimum	50 ppm	SJNNV	10 min	inactivation	Arimoto et al.* 1996
Chlorine	25 ppm	SBNNV-15°C DW	30 min	inactivation	Frerichs et al. 2000
	25 ppm	SBNNV-15°C +5% FBS	30 min	90 %	Frerichs et al. 2000
	50 ppm	SBNNV-15°C – DW	5 min	inactivation	Frerichs et al. 2000
	50 ppm	SBNNV-15°C +5% FBS	5 min	no inactivation	Frerichs et al. 2000
	50 ppm	SJNNV	10 min	inactivation	Arimoto et al.* 1996
	100 ppm	SBNNV-15°C - DW	5 min	inactivation	Frerichs et al. 2000
	100 ppm	SBNNV-15°C +5% FBS	15 min	99%	Frerichs et al. 2000
Iodophor	25 ppm	SBNNV-15°C -DW	30 min	inactivation	Frerichs et al. 2000
	25 ppm	SBNNV-15°C +5% FBS	30 min	no inactivation	Frerichs et al. 2000
	50 ppm	SJNNV	10 min	inactivation	Arimoto et al.* 1996
	50 ppm	SBNNV-15°C +5% FBS	30 min	99,7	Frerichs et al. 2000
	100 ppm	SBVERV-475-98	10 min	inactivation	Maltese and Bovo 2001
	100 ppm	SBNNV-15°C +5% FBS	5 min	99,99%	Maltese and Bovo 2001
Ozone	0.1 ppm	SJNNV	2,5 min	inactivation	Arimoto et al.* 1996
	0.5 ppm	SJNNV	30 sec	inactivation	Arimoto et al.* 1996
	3.6 ppm	SBVERV – MW	10 min	no inactivation	Bovo (unpublished)
	0.3 mg	HHVERV- MW	30 sec	egg disinfect.	Grotmol and Totland 20
	0.5 ppm	SBVERV – M.W.	-	44%	Borghesan et al. 2002.
	1.5 ppm	SBVERV – M.W.	-	99.93%	Borghesan et al. 2002.
	2 ppm	SBVERV- M.W.	-	99.99%	Borghesan et al. 2002.
	0,5 ppm	SBVERV- F.W.	-	99.99%	Borghesan et al. 2002.
	1,5 ppm	SBVERV- F.W.	-	99.99%	Borghesan et al. 2002.
	2 ppm	SBVERV-F.W.“	-	99.99 %	Borghesan et al. 2002)

* Residual infectivity was tested by exposing treated and non treated samples on susceptible larvae.

Priority research needs regarding disinfection and survival

In order to demonstrate the efficacy of egg disinfection in marine aquaculture, future research projects should address the following topics:

1. Experimental infection of spawners and identification of VER/VNN virus in sexual tissues and products.

2. Efficacy of iodophors in marine water and effect of high salinity and alkaline pH.

3. Possibility to disinfect eggs in freshwater environment.

4. Toxicity investigation of most common eggs disinfectant used (ozone, iodophors, glutharaldehyde) for eggs of different fish species at different development stages and temperatures, comparative egg mortality and teratogenic effects in fry.

5. Disinfection trials of eggs obtained from naturally and experimentally infected parents; long term observation in juvenile mortality.

Treatments must be considered for the most important reared species because some evidence exists to suggest differences between species.

Infectious pancreatic necrosis (IPN)

As shown in Work Package 1, there is clear evidence in the scientific literature for vertical transmission of IPNV via the fertilised egg of trout species and that disinfection of the eggs does not prevent this suggesting the virus is within the fertilised egg. However, it is not yet certain what the actual mechanism is although in general it seems that vertical transmission to the gametes is proportional to the amount of infectious virus in the gonads. Some studies have failed to demonstrate that the virus enters the egg whilst within the female parent but other work has provided some evidence for the virus entering the egg via attachment to the spermatozoa of males that are IPNV carriers. Further research is needed to determine whether vertical transmission in trout occurs through true intra-ovum transmission via the embryo as a result of virus entering the egg either directly from the female IPNV carrier or via the sperm of carrier males at fertilisation, or if it can occur by either route.

In contrast to trout, the evidence for vertical transmission in Atlantic salmon is sparse and inconclusive and there is very little published information to indicate that vertical transmission of IPNV occurs in any non-salmonid freshwater species. Furthermore, no evidence has been presented to suggest that vertical transmission occurs in marine fish species known to be susceptible to IPNV infection.

Virus survival outside the host

The published scientific literature on IPNV provides a significant amount of data on the stability/lability of the virus in the aquatic environment, particularly the effects of salinity and presence of microbial flora on the rate of loss of infectivity. As early as 1965, it was shown that IPNV infectivity was stable in sea water at 22°C for 12 days, whereas survival was much less at lower salt concentrations (Moewus-Kobb 1965). The stabilising effect of saltwater on the infectivity of IPNV and the influence of the presence of microbial flora on the rates of loss of infectivity has since been clearly demonstrated. For example, Tu et al (1975) found that IPNV infectivity was quite stable in unfiltered natural stream and well waters for 10 days at 4°C and for 5 days at 15°C but after that the infectivity declined rapidly. At the same time, Desaultels and MacKelvie (1975) showed that in unfiltered freshwater from a trout hatchery undergoing an IPN outbreak, 99% of virus infectivity was lost within 10-12 weeks at 4°C. In contrast, in filtered seawater, the loss of infectivity at 4°C and 10°C after 10 weeks was negligible and even after 5-6 months the loss was less than 99%. In commenting that IPN infectivity is more stable in seawater than in freshwater, the authors made the point that had the freshwater samples been filtered to remove microbial activity before storage, the virus survival may have been higher.

Wedemeyer et al (1978) demonstrated that from an initial titre of 10^5 $TCID_{50}$/ml, IPNV retained infectivity for at least 8 weeks in phosphate buffered distilled water and also in soft and hard natural lake waters at 10°C. In a later study on the effect of both water temperature and salinity on the survival of IPNV in the aquatic environment, Toranzo and Hetrick (1982) using natural marine, estuarine and freshwater samples showed that the virus had greatest stability in estuarine water (pH 7.8, salinity 6.5 ppt) at 15°C. At this temperature, a 99.9% reduction in infectivity took over twice as long (27 days) as at 20°C (12 days). In contrast, in full seawater (pH 8.0, 29.0 ppt) there was no significant difference in the time for a 99.9% reduction at these two temperatures (17 and 14 days respectively). The virus was least stable in freshwater at 20°C with a 99.9% inactivation in only 9 days, compared to 17 days at 15°C. Interestingly, the authors noted that the time of maximum bacterial concentration in the water correlated with the highest rate of inactivation of the virus.

The influence of physical treatment of estuarine water on survival of IPNV was clearly demonstrated by Toranzo et al (1983) who showed

that the time required for a 90% reduction in infectivity of the virus was at least four times longer in filter-sterilized or autoclaved estuarine waters (pH 7.6, 11.5 ppt) than in untreated water, indicating that the presence of viable microbial flora in the water enhanced the rate of virus inactivation. These effects were confirmed by Kamei et al (1987) who consistently found that IPNV infectivity was reduced by 99-99.9% in untreated seawater and sediment-containing water within 14 days at 15°C whereas in filtered or autoclaved seawater there was very little loss of infectivity. The authors concluded that the rate of IPNV inactivation in water is increased by the presence of bacterial activity. In a comprehensive study on the comparative stability of fish viruses, Ahne (1982) found that IPNV infectivity was not completely lost in tap water at 10°C after 231 days whereas the virus lost all infectivity within 21 days when suspended in river water. Ahne concluded that this effect is attributable to the strong bacterial growth present in the unfiltered river water. However, in pond sludge, at 4°C and 10°C IPNV infectivity was lost relatively slowly indicating that pond sludge of a virus-infected farm might act as a source of infection over long periods.

Overall on the evidence above, it can be concluded that IPNV is more stable in the marine environment than the freshwater environment and that the higher the microbial content of the water, the faster is the loss of infectivity in both aquatic environments. The rate of infectivity loss also increases with increasing water temperature.

Susceptibility of the pathogen to disinfection procedures

There have been numerous studies to determine the susceptibility of IPNV to inactivation by a range of physical and chemical disinfecting agents. These provide a useful indication of the conditions required for disinfecting IPNV contaminated hard surfaces of tanks, hatchery troughs and farm equipment with chemicals, the treatment of potentially-contaminated water supplies with UV light or ozone prior to hatchery use or discharge to the environment, and the use of iodophors for disinfection of salmonid eggs. Several studies have shown IPNV to be more resistant than other salmonid fish viruses and many treatments have failed to completely inactivate the virus infectivity even after prolonged treatment periods or high dosages. The main findings of a variety of published studies are summarised in the following table 8.

Although iodophors have been shown to be highly effective as disinfectants for IPNV in-vitro, studies have demonstrated that iodophor treatment of embryonated trout eggs at the eyed stage does not prevent vertical transmission of the virus (Bullock et al. 1976). Neither was vertical transmission prevented by the application of an iodophor (Romeiod) at concentrations from 25 to 200 ppm iodine (the maximum tolerated by the eggs) during water hardening of trout eggs for 45 minutes following fertilisation with IPNV-contaminated sperm (Dorson and Torchy 1985, Dorson et al. 1997) – the virus was subsequently isolated from all the hatched fry. The authors speculated that IPNV is protected by its microenvironment in the egg and that the iodine molecules are fixed and neutralised by the proteins of the hardening eggshell as soon as they pass through it. In contrast, Ahne and co-workers (1989) observed a dose-dependent reduction in the number of infected individual fry hatching from IPNV-contaminated fertilised trout eggs treated with low concentrations of an iodophor (Actomar K30) shortly after fertilisation. Eggs from IPNV-free females were contaminated with the virus 30 minutes after fertilisation with IPNV-free sperm by the dry method, then 1 hour later samples of the eggs were treated for 10 minutes with iodophor solution at a range of concentrations. At 11 days post-hatch, the virus was detected in 75 -81% of fry from untreated eggs, but this was reduced to 56.2%, 39.7%, 22.5%, 11.3% and 1.5% of fry from eggs treated with 0.5, 1, 2, 3 and 4 ppm iodophor respectively. Although at first sight these results look promising, it is important to remember that the study involved eggs that were artificially contaminated with IPNV more than an hour after fertilisation and this is not a true reflection of what happens in natural egg-associated transmission of IPNV. Further studies are required using eggs and sperm from naturally infected parents or use of sperm artificially contaminated with the virus to fertilise the eggs.

Attempts have also been made to prevent vertical transmission of IPNV by pre-treatment of the sperm with iodophor prior to fertilisation and by fertilisation of trout eggs in the presence of iodophor (Dorson et al. 1996). Pretreatment of sperm for just a few seconds resulted in low to nil fertilisation rates except when the sperm was in large excess but in that case the iodine failed to inactivate the virus. Fertilisation of the eggs in the presence of iodophor followed the procedure used by some fish farmers (eggs drained free of ovarian fluid, covered with the iodophor solution and sperm added immediately). Dorson and co-workers found that for iodophor concentrations of 15, 25 and 35 ppm at a pH in the range 5-8, less than 10% of the eggs were fertilised and this only increased to 69% at pH 9 with 25 ppm iodine. However, inactivation of the virus decreased substantially at pH values above 7 and the authors commented that virucidal efficacy could never match with safety for the sperm at any pH. So, at the present time, there is no known practical method of disinfecting IPNV-contaminated sperm by iodophor or any other treatment.

Table 8: Overview of studies on inactivation of infectious pancreatic necrosis virus (IPNV)

Method	Dose	Comment	Contact time	Outcome/ titre reduction	Reference
Heat	55°C		22 hours	incomplete	MacKelvie and Desautels 1975
	60°C		30 min	99.99%	MacKelvie and Desautels 1975
	70°C		2 hours	inactivated	Whipple and Rohovec 1994
	80°C		10 min	inactivated	Whipple and Rohovec 1994
UV-C	10-30 J/m^2			99%	Yoshimizu et al. 1986
	3300 J/m^2	tap water		80%	Maisse et al. 1980
	1200 J/m^2	freshwater		99.9%	Øye and Rimstad, 2001
	1220 J/m^2	lake, estuarine and sea water		99.9%	Liltved et al. 1995
	1500–2000 J/m^2	PBS		99.9%	Sako and Sorimachi 1985
Formalin	0.025%	4°C, PBS	14 days	incomplete	MacKelvie and Desautels 1975
	0.2%	21°C	60 min	incomplete	Elliott and Amend 1978
	2%	aqua dest.	5 min	inactivated	Vestergard-Jørgensen 1973
	3%		5 min	inactivated	Ahne 1982
NaOH	pH 11.9	8-10°C,	5 min	inactivated	Ahne 1982
	pH 12.2		10 min	inactivated	Vestergard-Jørgensen 1973
Acid	pH 2.0	4°C, KCl-HCl	5 weeks	incomplete	MacKelvie and Desautels 1975
	pH 2.5	8-10°C, HCl	60 min	ineffective	Vestergard-Jørgensen 1973
	pH 3	10°C, HCl	60 min	ineffective	Ahne 1982
	pH 4 citric phosph. buffer	22°C		ineffective	Whipple and Rohovec 1994
	pH 3.8-4.3 fish silage	22°C		ineffective	Whipple and Rohovec 1994
Propanol	90%	15°C, PBS	90 min	ineffective	Inouye et al. 1990
Ethanol	90%	15°C, PBS	60 min	ineffective	Inouye et al. 1990
Methanol	80%	15°C, PBS	30 min	inactivated	Inouye et al. 1990
Phenol	5%	15°C, PBS	5 min	inactivated	Inouye et al. 1990
Cresol	5%	15°C, PBS	5 min	inactivated	Inouye et al. 1990
Iodophor	32 ppm	PBS	5 min	inactivated	Amend and Pietsch 1972
	35 ppm	21°C, aqua	5 min	inactivated	Desautels and MacKelvie 1975
	16 ppm	dist	5 min	inactivated	Elliott and Amend 1978
	10 ppm	15°C, PBS	2.5 min	inactivated	Inouye et al. 1990
	2.5 ppm	15°C, PBS	60 min	inactivated	Inouye et al. 1990
Chlorine	0.1 mg/l	10°C	1 min	inactivated	Wedemeyer et al. 1978
	0.2 mg/l	10°C, soft water	10 min	inactivated	Wedemeyer et al. 1978
	0.2 mg/l	10°C, hard water	10 min	ineffective	Wedemeyer et al. 1978
	0,7 mg/l	10°C, hard water	2 min	inactivated	Wedemeyer et al. 1978
	4 mg/l	21°C titre $10^{4,5}$	5 min	inactivated	Elliott and Amend 1978
	4 mg/l	15°C PBS	2.5 min	inactivated	Inouye et al. 1990
	25 mg/l	20°C titre $10^{5,0}$	30 min	inactivated	Desautels and MacKelvie 1975
	30 mg/l	10°C tapwater	2 min	99%	Ahne 1982
	40 mg/l	20°C titre $10^{7,5}$	30 min	inactivated	Desautels and MacKelvie 1975
Ozone	70 mg/h*l	10°C soft w.	10 min	inactivated	Wedemeyer et al. 1978
	90 mg/h*l	10°C, hard w.	10 min	Inactivated	Wedemeyer et al. 1978
	0.11 mg/l	SW	1 min	99.7%	Itoh et al. 1997
	0.1-0.2 mg/l	Lake, estuarine, SW	30-60 sec	99.99%	Liltved et al. 1995

Priority research needs regarding pathogen survival and disinfection

1. Standardised methods need to be developed to facilitate more accurate comparison of the rate and extent of inactivation of IPNV in natural marine, estuarine and fresh water exposed to physical and chemical disinfecting agents. These standardised methods should then be used to provide quantitative data on the rate of inactivation of the virus by physical or chemical agents under conditions typical of salmonid and marine fish hatchery environments.

2. The studies of Ahne et al. (1989) that showed a significant reduction in the number of infected individual fry hatching from trout eggs fertilised then treated soon after with iodophor need to be corroborated and extended to eggs and sperm from naturally infected parents or use of sperm artificially contaminated with the virus to fertilise the eggs. The research should also include the iodophor water-hardening conditions that have been found to be effective for preventing vertical transmission of IHNV in salmonids.

3. The toxicity and teratogenicity of iodophors for embryos in eggs disinfected during fertilisation or water hardening will need to be determined over a range of conditions including temperature, pH, and salinity of the hardening water and the concentration of iodophor.

Infections with Gram-positive bacteria

Bacterial kidney disease

(BKD, Infection with *Renibacterium salmoninarum*)

It was stated in WP1 that vertical transmission of *Renibacterium salmoninarum* was no longer a matter of discussion. All sources of information, field observations, experimental approaches, direct microscopy and, as a practical confirmation, the striking results obtained after control and managing provisions had been introduced are clearly indicative of in ovo contamination of fry, and let little doubt about the reality of intra-ovum infection.

Pathogen survival outside the host

Several authors have established that the causative bacterium is tightly adapted to fish colonisation and is not likely to survive for a long time in aquatic environment. While Evelyn (1993) noticed a quick disappearance of *R. salmoninarum* in natural habitats, namely when bivalve populations were present, Austin and Rayment (1985), and Balfry et al. (1996) could no more detect any cultivable bacteria after experimental exposure for 4 days and 14 days in stream water and sea water, respectively. In sterile freshwater the survival did not exceed 28 days. More recent experiments by Hirvelä-Koski (2004) suggested, in fact, that starvation forms could become detectable in small numbers after longer incubation times, and then could persist for several weeks. Nevertheless, the bacterial population is severely affected by external adverse conditions that should favour disinfection and make it very effective in routine practice.

Susceptibility of the pathogen to disinfection procedures

Although disinfection procedures have been early introduced in fish culture management to limit the occurrence or introduction of infectious agents, scientific references to disinfection for controlling bacterial kidney disease are scarce. Clearly, the promising development of eggs or spawning fish medicinal treatment to reduce the infection rate in progeny and to strengthen large scale health control programmes received greater attention. As the causative bacterium was considered to occur regularly in the feral populations of salmon captured and used for sea-ranching, disinfection could not appear as a really convenient control method. It appears that the only specific studies to assess the bactericidal effect of usual

disinfectants on *R. salmoninarum* were conducted with chlorine by Pascho et al. (1995), Pascho and Ongerth (2000) and Hirvelä-Koski (2004). The results were quite convincing, the bacteria being inactivated at 99 % with 0.05 mg /l in 1 to 18 s. Fluctuations may be observed depending upon the quality and pH of water, and in practice the recommended concentrations may vary from 10 to 200 mg/l.

Egg disinfection is better documented, and many reports have been produced about the use of iodine derivatives to limit the contamination with the BKD agent. Initially tested in 1972 by Ross and Smith, who could assess in vitro that *Renibacterium* was inactivated in 5 min with 25 mg /l of Betadine® or Wescodyne®, the effectiveness of iodophors use in field situation was questioned by Bullock et al. (1978). Even when concentration in active product was increased up to 100 mg /l, a partial effect of treatments was repeatedly noticed (Elliott et al., 1989). This was eventually explained by the demonstration by Evelyn et al. (1984, 1986), that bacteria located inside the eggs could not be inactivated by iodophors after water-hardening process had occurred. It is generally accepted, however, that before water-hardening, iodophor disinfection at high doses (500 mg /l) eliminates the bacteria colonising egg surface (Evelyn et al., 1984; Armstrong et al., 1989).

Among other possibilities, neither ozone nor UV irradiation seem to have been tested on salmonid eggs. From preliminary data conducted in marine species, ozone could prove useful to eliminate surface contaminants including bacteria (Sugita et al., 1992), but toxic effects should better be documented first (Grotmol et al., 2003). Hydrogen peroxide, conversely, was shown to be a potent disinfectant of egg surface (Waterstrat and Marking, 1985; Schreier et al., 1996). Schreier et al. (1996) and Gaikowski et al. (1998) agreed about a daily exposure of 15 min at 500 µg /l, taking care of the possible toxicity to certain fish species according to temperature (Rach et al., 1997). Hydrogen peroxide has been mainly been used to treat fungal infections, in fact, and adaptation of these prescriptions have not yet been tested in renibacteriosis control strategy. Use of hydrogen peroxide has not been noticed in the scientific literature, although a successful test is reported on a commercial company website (Antec International 2003; http://www.antecint.co.uk/main/virkaqua.htm).

Priority research needs regarding pathogen survival and disinfection

1. In order to improve or complete our knowledge about the susceptibility of *R. salmoninarum* to disinfection, introduction of new disinfectants or disinfecting procedures would deserve further testing. Two related questions are the toxic effects of these methods on eggs or egg hatching, and the usefulness of facilities and material disinfection as a complementary measure to parent fish control and sanitation.

2. Another point of significance, considering the future needs for risk analysis, is to get more quantitative data about the survival of *R. salmoninarum*, both in the different compartments of the environment and in fish tissues, including gonad and eggs. As yet, few figures are available on this special point.

Flavobacterium psychrophilum infections (rainbow trout fry syndrome, RTFS)

Vertical transmission of *Flavobacterium psychrophilum* is generally accepted as a very likely mode of contamination. It must be recalled, however, that no definitive evidence, namely the direct observation of bacteria inside the eggs, was ever produced as it was done with *R. salmoninarum*, and that not all workers were able to obtain positive results when attempting to detect bacterial DNA inside the eggs using PCR.

Pathogen survival outside the host

Investigations on the survival of *F. psychrophilum* in natural or farm environments were undertaken lately, in fact, and long hampered by difficulties in the bacterium culture. Recent improvements in culture media formulation opened to new approaches. Despite a very low ability to survive in distilled water (Michel et al., 1999, Vatsos et al., 2003), it was found that *F. psychrophilum* could adsorb and survive about one month on the egg surface, and 10 to 30 days in stream water (Vatsos et al., 2001; Kondo et al., 2002). In stream water, the ability to survive was accompanied by the formation of viable but non-cultivable (VNC) forms expressing variations in morphological and antigenic properties (Vatsos et al., 2003). Inoculating sterilized water microcosms and maintaining them for longer periods of time, Madetoja et al. (2003) did not notice VNC but established that after a pronounced phase of decrease the bacterial population was stabilized, and even slightly reactivated, permitting the survival of culturable cells for over 300 days. Such properties should be considered before advising control procedures based on disinfection.

Susceptibility of the pathogen to disinfection procedures

There are still less experimental data published on *F. psychrophilum* than on *R. salmoninarum* to support practical application of disinfection procedures to flavobacteriosis. Papers really focusing on *F. psychrophilum* disinfection methods apparently do not exist in scientific literature. Some experimental works, actually, were published about the susceptibility of other *Flavobacterium* species to bactericidal products, so allowing tentative extrapolations. Quaternary ammonium compounds became very popular after Rucker (1948) demonstrated their efficacy in limiting the proliferation of bacterial gill disease agents. Wood, in 1974, reviewed several diseases caused by "myxobacteria", among which columnaris and low temperature diseases. In almost every case he pointed out the interest of Hyamine®, when used both for fish treatment and materials disinfection, but *F. psychrophilum* was the only species for which an antibiotic treatment was preferred! Later, in a comparative study of several commercial quaternary ammonia products, Dorson and Michel (1987), noticed that *Flavobacterium spp.* strains appeared generally more susceptible than most of the other fish bacteria tested, even though the efficacy of fish treatment doses was questioned in regard to toxicity thresholds, so confirming former reservations of Rucker et al. (1949), and Wood (1968). Thus, we have strong reasons to consider that quaternary ammonia are excellent products to inactivate *F. psychrophilum* in fish farm facilities, but should no be used for external treatments. Our documented background, however, does not deliver much more.

Use of iodophors thoroughly studied and recommended by Amend (1974) for viruses and bacteria removal of egg surface, was soon considered in cold-water disease infection (Holt 1972, Schachte, 1983). According to Brown et al. (1997), using iodophors at 100 ppm, egg surface disinfection is completed at 100 % in 60 min, and at 98 % in 30 min. Lower doses may be effective in vitro, but remain unsuitable for egg disinfection purpose (Kumagai and Takahashi, 1997). It may be concluded that, providing convenient protocols are respected and internal contamination has not occurred, egg disinfection is a valuable practice to limit the transmission of the disease. Opportunity was taken of all these different observations in several experimental studies aiming at the demonstration of true vertical transmission of the disease (see WP1 and Kumagai et al., 1998). At last, there are some indications that hydrogen peroxide, also, could offer interesting possibilities for trout egg disinfection. In recent tests, it proved effective for treating bacterial gill disease, a condition caused by another Flavobacteriaceae, *F. branchiophilum* (Rach et al. 2000).

Priority research needs regarding pathogen survival and disinfection

Although recent investigations were developed about the survival potency of *F. psychrophilum* in the environment and outside the host, much remains to do before understanding the ecology of the bacterium. The suspected existence of viable non-culturable forms will probably increase the difficulty, and should not be disregarded in future studies on the feasibility and efficacy of disinfection. As for BKD, understanding the physiology of bacterial cells and getting quantitative information about their infective potency under different micro-environmental conditions would represent a key step to promote the development of risk assessment strategies.

Piscirickettsiosis
(Piscirickettsia salmonis)

In work package 1, experimental studies reporting infected offspring after crossing infected and non-infected parental fish, and the demonstration of *P. salmonis* attached to and apparently penetrating the salmonid egg surface (Larenas et al. 2003) was reviewed. The conclusion was that vertical transmission of *P. salmonis* including so-called "true vertical transmission" should now be considered scientifically established. However, like discussed by other reviewers (Lannan et al. 1999, Mauel and Miller 2002) there is a discrepancy between the endemic nature of this infection among Chilean salmonid broodfish and the scarcity of reports about disease occurrence in the hatcheries and fresh water farms receiving eggs and embryos. Poor ability of the pathogen to survive outside the host and its sensitivity to commonly practised egg disinfection methods may offer an explanation for this apparent contradiction.

Pathogen survival outside the host

Like viruses and unlike bacteria, *P. salmonis* is unable to grow in vitro outside living cells. Most of the closely related, so-called rickettsia-like organisms (RLO's) known from terrestrial animals require transmission through living vectors, mainly insects but both field observations and experimental data suggested that direct transmission via the aquatic environment did occur. In order for such transmission to be of major importance in the epidemiology of the disease, the organism must be able to survive extracellularly. This aspect of piscirickettsiosis epidemiology has, with the exception of one study referred to below, been poorly investigated.

In a series of experiments, *P. salmonis* grown on CHSE-214 cell culture was maintained at 5, 10, 15 or 20°C and kept for up to 4 weeks in cell culture media or various types of filter-sterilised water (Lannan and Fryer, 1994). Infectivity of the treated organisms to uninfected fish cell cultures was titrated every 2 days. When suspended in spent or fresh cell culture medium, a temperature-dependent drop in titre was observed but the piscirickettsia maintained their cell culture infectivity for up to 21 days at 5 and 10°C. When cell culture medium was removed and the organisms were re-suspended in full-salinity sea water, infectivity was retained for 12-15 days dependent of temperature, not significantly differing from the survival time seen in fresh cell culture medium. However, suspension in either of the tested fresh water preparations immediately rendered the cells unable to infect the cell cultures. The authors concluded that the rapid inactivation in fresh water might explain why the piscirickettsiosis, unlike in the marine environment, is nearly absent in freshwater sites.

There are no clear indications which organism(s) may constitute the natural host(s) of *P. salmonis*, nor which species or environmental compartments may serve as epidemiological reservoirs. It is therefore impossible to hypothesise on the risk for egg or embryo contamination from such sources. PCR findings suggesting the presence of *P. salmonis* or closely related organisms in bacterioplankton from North American coastal waters has, however, been reported (Mauel and Fryer, 2001). One experiment has been presented to suggest that *P. salmonis* may occasionally be recovered from mussels after exposure to infected seawater (Smith et. al 1999). Recovery was very scarce, however, giving no indication for accumulation and long-term maintenance of piscirickettsia in these bivalves.

Disinfection

There is remarkably little information available on the susceptibility of *P. salmonis* to common disinfection procedures. In a study presented at the International Symposium on Aquatic Animal Health in Baltimore, Palm et al. (1998) reported that 5 ppm chlorine added to fresh water did result in complete inactivation, and this concentration is also being recommended for inactivation by Dr. Pedro A. Smith, University of Chile (pers.comm.). One should, however, remember that there is a very rapid inactivation also by fresh water alone. We have found no information relating to disinfection studies with *P. salmonis* in sea water.

Priority research needs regarding pathogen survival and disinfection

The following information is needed to further develop and optimise risk management and risk control measures for vertical transfer of *P. salmonis*:

1. Survival of *P. salmonis* in waters of various salinity and temperature, with and without the presence of serum or other organic materials.

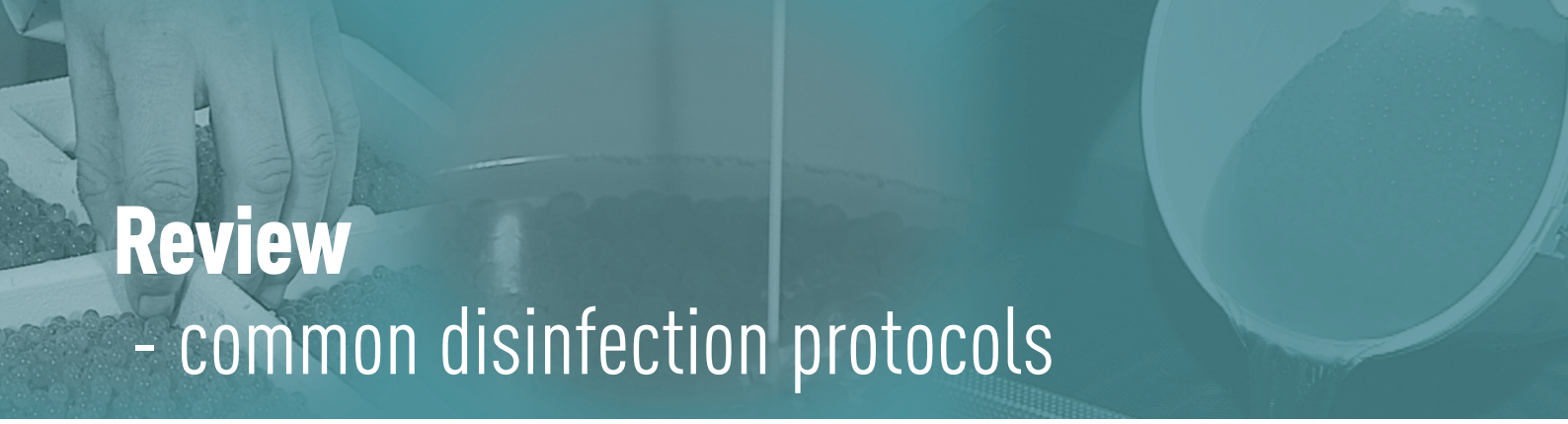
2. Survival of *P. salmonis* inside cultured macrophages.

3. Survival of *P. salmonis* on the surface and inside fertilised eggs obtained from parents shedding the organism with their sexual products.

4. Sensitivity of *P. salmonis* to UV light exposure.

5. Sensitivity of *P. salmonis* to common egg surface disinfectants of various salinities and at various temperatures.

6. Transmission experiments using experimentally infected gametes or embryos, with and without subsequent disinfection procedures (expansion of work initiated by Larenas et al. 2003).



Review
- common disinfection protocols

Chile

According to fish health regulations embryonated eggs shall be disinfected in the farm where the eggs are to be hatched. Disinfection shall be carried out in 100 ppm free iodine at pH between 6 and 8. Submerge the eggs in the iodized solution during 10 minutes. Thorough rinsing of the eggs shall be done prior to and after the disinfection. Application shall not exceed 2000 eggs per litre of disinfectant solution. Use an abundant amount of solution and replace it when it acquires a light-yellow tone and before colour disappears. The resulting liquid residues must be disposed of in a manner non-detrimental to the environment. The packaging used during transportation of the eggs must also be disinfected, or better yet, destroyed through a procedure that prevents any sanitary hazard or contamination in the water and/or other fishes of the place of destination. Farms where hatching takes place must notify the nearest Service office on the date and time of disinfection with at least 24 hours in advance. It is not mandatory or generally practiced to disinfect newly fertilized eggs (Annex 1). (Information provided by Arne Storset and Ricardo Enriquez).

Denmark

Disinfection of fish farms and salmonid eggs are regulated according to " Veterinærdirektoratets cirkulære af 27. august 1986 om rensning og desinfektion af ferskvandsdambrug, m.v."

Cleaning and disinfection of fish farms:

1. Drain whole farm for water, remove mud and clean.
2. Disinfect by using quick lime, min 1/2 kg/m^2.
3. If farm cannot be kept drained for whole fallowing period (due to high groundwater level) the farm must be emptied at least twice and treated with lime.
4. Tanks, equipment etc are cleaned by high pressure, or brush using appropriate detergents, followed by disinfection with formalin (2%) or iodophor 2-3%.

Eggs from salmonids:

Eyed eggs: Disinfection in neutral iodophor 50 -100 ppm for 10 min followed by rinsing in clean water. Prevention of after-contamination must be ensured.

For Export: All eggs must be disinfected before shipment and placed in new or properly cleaned and disinfected vials.
For inland use: No official requirements, but it is common practise to disinfect eyed eggs.
It is not common practise to disinfect green eggs in Denmark.

Transport vehicles and equipment:
All vehicles and equipment used for transport of live fish and products from aquaculture must be properly cleaned and disinfected after use using formalin or iodophor. Water must only be discharged into sewerage with outlet directly to seawater or for percolation.

England and Wales

Importers of eyed eggs of salmonids are issued with specific instructions on the disinfection of fish eggs (DOF 4, Annex 3). There are no mandatory disinfection guidelines in England and Wales.

Immediately after the ova have been taken from their transport packaging and containers, all packaging and containers must be safely disposed of and not be recycled or re-used and guidance to avoid contamination of hatcheries from transport materials is being given.

For disinfection, a 50 ppm solution of the iodophores Wescodyne or Buffodine is recommended for use, but no minimum disinfection time is being stated. It is further stated that at a concentration of 50 ppm available iodine, Wescodyne is relatively non-toxic to fertilised trout ova and, with an exposure time of 10 minutes and a pH above 6.5, there is a considerable margin of safety. However, at the strength recommended the solution is considered being highly toxic to unfertilised ova (green eggs) and to live fish.

Faeroe Islands

According to information received, the fish egg producers in the Faeroe Islands use the standard procedures used in Norway (see Annex 5). (Information provided by Mr. Peter Østergård).

France

No official text to specify technical procedures is available. Fish farmers generally refer to technical publications or leaflets edited by professional associations. The most common protocol is iodine

disinfection of eyed eggs at 50 or 100 ppm for 10 min; followed with neutralisation of the product with sodium thiosulphate (commercial products are supplied with notices). Iodophor disinfection during or immediately after egg fertilization is not commonly used since negative experimental results have been published in French literature (Dorson, 1989; Dorson et al., 1996). (Information provided by Dr. Christian Michel).

Iceland

For disinfection of eggs from Atlantic salmon and other anadromous or freshwater species, iodophor (Buffodine) solutions are being used. All eggs are disinfected immediately at stripping, and in some cases, the same procedure is being repeated at the eyed egg stage (mandatory when exporting eggs). 100 ppm Buffodine solution is being used, that is 100 ml Buffodine in 10 litres of water. Disinfection time is at least 10 minutes. The entire egg container must be submerged in the disinfection solution during the entire disinfection period.

Surface disinfection of eggs of marine species such as Atlantic halibut, turbot, cod and spotted wolffish is relevant in Icelandic aquaculture. Eggs from these fish are being disinfected with glutaraldehyde. Eight ppm solutions (9,6 ml concentrated glutaraldehyde per 6 litres of water) are being used. Eggs are normally transferred directly from the fish into the disinfection solution, and kept there under gentle mixing for minimum 5 minutes. After the treatment, the eggs are being rinsed several times in fresh seawater; total rinsing time is minimum 5 minutes. (Information provided by Gisli Jonsen/Sigurdur Helgason).

Ireland

No written instructions are established. However, a general recommendation is to use an iodine based disinfectant (usually Buffodine®) and carry out the disinfection in accordance with the instructions supplied. In case of imported ova or ova transferred from another site, to burn all packaging as soon as possible after arrival avoiding surfaces and water supply at the receiving farm. (Information provided by Fiona Geoghegan).

Italy

No compound has so far been licensed specifically for the control of fish egg contaminating pathogens, but because of the lack of any evident risk for human health, the use of iodophors is not prohibited and is being widely used in trout farms.

Salmonid fish

In the Trento Province where the majority of Italian trout farm hatcheries are situated, the local veterinary authority has adopted an official disinfection protocol based on the use of iodophors during water hardening, according to the method described by LaPatra (Annex 4).

The implementation of this method on the majority of the egg farms significantly reduced the number of IHN outbreaks in hatcheries. Furthermore embryonated eggs, when moved from one farm to another, are submitted in the arrival farm to iodophor disinfection by immersion in 100 ppm iodophor solution for 10 min.

Marine species (Sea bass and sea bream)

No official data are available and no disinfection protocol has been officially established, nevertheless the most important hatcheries, following the serious losses caused by VER/VNN all over the Mediterranean area during 1995, has adopted their own disinfection protocol based on the use of iodophors. Eggs are submitted to iodophors disinfection immediately after harvesting. Disinfection is performed by immersion of egg in 50-100 ppm iodophor for 10 minutes. Unfortunately no information is available or has been published on the efficacy of iodophor treatment. (Information provided by Guiseppe Bovo).

Norway

The most widely used disinfectant is the iodophor Buffodine®. The eggs are disinfected at 100 ppm iodophor for 10 minutes (Annex 5). To postpone the water hardening process until the disinfection is completed, the stock solution of Buffodine is mixed in 0,9 % NaCl. See also: http://www.drydenaqua.com/chemicals/disinfectants/buffodine.htm). The Hatchery Regulations does not impose compulsory egg disinfection at the eyed eggs stage. But if the eggs are to be transferred from one site to another, egg disinfection is an industry standard and part of Good Manufacturing Practice. The eggs are disinfected prior to dispatch or when the eggs arrive at the destination hatchery - preferably the latter.

Scotland

In Scotland, there is a policy of controlling vertical transmission of IPN in salmon such that brood fish are tested at time of stripping and eggs from parent fish that test positive are not permitted to be used. The FRS Fish Health Inspectorate audit hatcheries to ensure satisfactory procedures and separation of stocks etc are in place. Guidance notes to salmon farmers which contain information on the disinfection of eggs are produced.

It is recommended that pre-hardened eggs are disinfected with iodophor-based disinfectant as soon as possible after fertilisation following the protocol given in the OIE International Aquactic Animal Health Code. In addition, it is specified that the disinfectant must not be used for more than one pool of eggs (individual pairing or up to 5 females and 5 males) and it must retain its colour after disinfection is complete. Disinfection of eyed eggs prior to movement of the eggs to another water supply is also recommended.

It is not known that the marine finfish sector of the industry routinely disinfect eggs of cod or halibut given the lack of a satisfactory, safe protocol. (Information provided by Pauline Munro).

Sweden

In Sweden, disinfection of ova is mentioned in Regulations given by the Ministry of Agriculture (Jordbruksverket) and Ministry of Fisheries (Fiskeriverket) such as that all ova shall be disinfected by a iodophor preparation. No direct instructions are written in the regulations as regards dosing, etc. However, the Fish Health Control Service ("Fiskhälsan") as always had a facts sheet for the purpose (Annex 6). In Sweden, only Buffodine® is available in the market and according to the Swedish regulations it is considered as a disinfection preparation and is thus in free trade without prescriptions. (Information provided by Ulf Peter Wickhardt)

Tasmania

Iodine is a potent disinfecting agent that is effective against a wide range of bacteria, fungi and viruses. Unfortunately iodine compounds together with their salts produced in aqueous solution are generally toxic and corrosive in nature.

Iodophor is the general name for any chemical compound in which a surfactant (wetting agent) acts as the carrier for the iodine. Iodophors have the advantage that they retain the cleansing properties of detergents whilst releasing iodine slowly and thus reducing its toxic effect. Fish eggs are relatively insensitive to iodophors at neutral pH but sensitive under acidic conditions. However, embryos and fry are comparatively sensitive to iodophors. As a consequence it is considered safe to use iodophors for most of the incubation period but they must be buffered to near neutral pH or be naturally neutral, such a povodine iodine. If iodophors is used at the very end of the incubation period it is possible that some iodine will wash off the surface of eggs and be harmful to any first hatch fry that may be present at the time.

Povodine-iodine is one of the most commonly utilised iodophors and uses a polyvinylpyrrolidone base that has the advantage of providing a neutral pH, is non-irritant and suitable for use in fish eggs. Common brand names of povodine-iodine solutions include 'Betadine', 'Minidine', 'Iovone', 'Vetadine' and 'PVP-Iodine Solution'. Most of these products come as a 10% W/V povodine-iodine solution, providing 1% available iodine in aqueous solution.

Products containing additives other than iodophores, i.e. surgical scrubs or dairy teat dips should be avoided for egg disinfectants. Technical procedures have been published (Annex 7). (Information provided by Rick Butler).

USA

Ionosphere disinfection is carried out both post-hardening as well as prior to (during) water-hardening. However, according to 1995 Fish Health Guidelines, all eggs taken by the U. S. Fish and Wildlife Service (USFWS) must receive a disinfection in active iodine (Argentyne; Argent Chemical Laboratories, Redmond, Washington) at 50 mg/L for 30 minutes prior to water-hardening and a subsequent 10 minute 100 mg/L post-hardening disinfection if they are shipped to another USFWS hatchery or aquaculture facility.

A standard procedure for iodophor water-hardening of salmonid fish eggs described by Scott LaPatra has become a commonly used practice among aquaculturists in North America (Annex 8). (Information provided by Wade A. Jodun, U.S. Fish and Wildlife Service, and Scott LaPatra).

Asia

In Asia fish egg trade is not big or does not take place at all. Thus there is so far no standard procedure for fish egg disinfectant in Thailand/Asia. There may be some hatcheries used fungicide such as trifluralin in hatcheries tank but again, not many hatcheries used because the hatching period is not long or fungal infection is not a major problem.

Formalin and iodine-base may be used more in shrimp hatcheries to reduce the viral contamination.

For shrimp some washing is done to remove virus particles. Hatcheries normally use Povidone Iodine (PVP) 50-100 ppm/10-60 seconds followed by rinsing with clean sea water. But for fish (carps and tilapia) egg disinfection is done in the hatching jars using various anti fungal agents and formalin.

The Philippines

According to information received, there may not be any regulation yet about egg disinfection in the Philippines, especially for trade purposes, but there's one published work:

- **Tendencia EA (2003).** Iodine disinfection of grouper *Epinephelus coioides* eggs. Bulletin of the European Association of Fish Pathologists **23** (4), 191-196.

Japan

Eggs are usually iodized for preventing virus infection. For example: povidone-iodine is used in 25ppm x 30-60min or 50ppm x 15min. (source: K. Hatai, Nippon Veterinary and Animal Science University, e-mail: hatai@scan-net.ne.jp)

For VNN - iodophor (povidone-iodine) and ozone are used to disinfect eggs in many hatcheries. Unfortunately it cannot prevent vertical transmission of viral diseases. Marine fish eggs are more sensitive to disinfectants than freshwater fish eggs; therefore, development of suitable disinfection measures is needed for marine fish eggs.. See also:

- **Sako M (1995).** Practical approaches to marine fish health problems in Japan, pp. 81-90. 1996. In: Kevan L. Main and Cheryl Rosenfeld (eds). Aquaculture Health Management Strategies for Marine Fishes. Proceedings of a Workshop in Honolulu, Hawaii, October 9-13, 1995.

(Information provided by Pedro Bueno [pedro.bueno@enaca.org], Celia R. Lavilla-Torres [celiap@aqd.seafdec.org.ph], Melba Reantaso [melba@goeaston.net], Pornlerd Chanratchakool [pornlerc@fisheries.go.th], Rohana Subasinghe [Rohana.Subasinghe@fao.org])

Summary

In WP1, evidence of vertical transmission for several finfish diseases was reviewed. For some of the diseases, this vertical transmission is suggested to be a result of a contamination of the egg surface ("egg associated transmission"). Such contamination may be effectively prevented by egg disinfection procedures, given that the disease agent is susceptible towards the applied disinfectant. The aim of the current work package has been to scrutinise the ability of the selected infectious agents to survive in the environment or on the egg surface, as well as their susceptibility to various disinfection procedures involved in treatment of eggs to prevent transmission of infectious agents from brood fish via eggs to offspring.

As regards survival outside the host, this issue is considered in relation to pH, effect of temperature such as cooling, freezing and heating, different environmental conditions such as fresh water, estuarine (brackish water and sea water, etc.)

pH
It has been shown that IHNV infectivity is considerably reduced below pH 5 or above 9. VHSV is also susceptible to both high and low pH resulting in reduction of infectivity within 3 hours at pH 3, while at pH 12, inactivation takes place within 5 – 10 minutes. Similarly, EHNV loose all infectivity below pH 2 or above pH 12. VNNV is also inactivated within a short period at pH ≥ 12.

ISAV seem to be relatively stable in the pH range 5 – 9, while at pH 4, inactivation is shown to take place within 30 minutes. Above pH 11 a 90% reduction has been demonstrated within 30 minutes.

Temperature
IHNV survives for more than 22 weeks at 4°C and even several weeks at 15°C, but is inactivated within hours at 32°C. At - 20°C IHNV has been shown to survive several years.

VHSV survives freeze drying and freezing at 20°C for years, several months at 4°C, approximately 4 weeks at 20°C, within hours at temperatures between 35 – 50°C and less than one minute at 70°C. In dry environment at 4°C, VHSV may survive for one week.

SVCV is rather unstable and is reported to survive less than 2 weeks at 23°C and less than half a year at 4°C while in tap water at 10°C, a survival time of 42 days have been reported. Like VHSV, the SVCV survive freeze drying and freezing for years.

EHNV in tissue culture or in fish tissues is still infective after being stored 2 years at – 20 –70°C. The EHNV keeps also infectivity for 97 days in distilled water and in infected cell cultures kept at 4°C, infectivity is maintained at least for 300 days.

ISA is reported to be stable for 14 days at 4°C and 10 days at 15°C, while 99,99% reduction infectivity was obtained at 56°C. Freezing and thawing seems not to reduce infectivity of ISAV.

VNNV is completely inactivated within 4 days at 37°C, while remaining full infectivity during 6 months at 15°C.

Aquatic environments
IHNV has been shown to survive in soft or hard fresh water for 7 weeks at 10°C, but only for 2 weeks in distilled water. Optimal survival temperature in fresh water is shown to be 15°C. Significant reduction in the IHNV titre takes place in brackish water, estuary water and seawater within 3 days.

VHSV has been reported to survive in tap water for 49 days at 10°C, while drying for 28 days at 4°C and 20°C

ISAV infected material exposed in sea water and fresh water at 10°C, is reduced after 24 – 48 hours although some infectivity was reported to exist after 48 hours. Experiments have indicated that ISAV may be able to survive for an extended period in sea water.

IPNV is reported to be stable in sea water for 12 days at 22°C while survival at lower salinities was much less. The infectivity of the virus is also quite stable in unfiltered river- and well water for 10 days at 4°C and 5 days at 15°C. The overall evidence on survival of IPNV reported, it can be concluded that the virus is more stable in the marine environment than in freshwater environment and that the higher the microbial content of the water is, the more rapid loss of infectivity occurs.

Renibacterium salmoninarum is not likely to survive for a long time in the aquatic environment as the bacterium is tightly adapted to the fish host. The bacterium disappears quickly in environments where bivalve populations are present. Under experimental conditions *R. salmoninarum* has been reported to survive no longer than 4 days and 14 days respectively in river water and sea water. In sterile freshwater survival has been demonstrated to be no more than 28 days. *Flavobacterium psychrophilum* has a low ability to survive in distilled water, but may survive for 10 - 30 days in river water and one month on the egg surface.

Piscirickettsia salmonis is unable to grow outside living cells. Suspended *P. salmonis* in seawater may survive for 12 – 15 days depending on the temperature, while a rapid inactivation occurs in freshwater.

Disinfection

There are numerous studies regarding survival and disinfection, but there are large variations in the amount of data for the different disease agents. For some agents (IHN, IPN, VER/VNN and ISA) there are several studies relating to pathogen susceptibility, while for others scientific data are scarce (EHN, *Flavobacterium* and *Piscirickettsia*). Although the susceptibility of some of the agents is thoroughly studied, a major drawback of these data is the lack of standardized test methods and test conditions. This could be the reason for some of the contradictory results found. The lack of consistency makes it difficult to compare results and give well founded disinfection recommendations.

Control of IHN by disinfection of the water supply is reported to have had limited success by using ozonation, ultraviolet light (UV), chlorination – dechlorination or addition of different types of germicides (ethanol, phenol, cresols, methanol, etc.). Iodine disinfection of egg surface have been shown to destroy 99, 98% of IHNV on the surface of green eggs and eyed eggs. Iodophores at a concentration of 100 ppm inactivate VHSV within 4 minutes under both clean and dirty conditions. No information exists as regards the effect of iodophores on the infectivity of EHNV or VNNV. Although iodophores have been demonstrated to be highly effective in in vitro studies, other studies have shown that iodophor treatment does not prevent vertical transmission of the viruses. High doses of iodophores used on salmonid eggs prior to water hardening have been shown to eliminate the bacterium on the egg surface. For flavobacteriosis (*F. psychrophilum*) egg disinfection using iodophores 100 ppm is 100 % effective within 60 minutes, while only 98% effective within 30 minutes.

Chemicals such as methylene blue, malachite green, benzalconium chloride and copper sulphate have no effect on VHSV or SVCV. Formalin on the other hand seems to inactivate both VHSV and SVCV within 5 minutes, while formalin treatment seems to have poor effect on VNNV.

Treatment of ponds and equipment with sodium hypochlorite 200 ppm is a recommended method for decontamination of EHNV.

Iodophores, chlorine- and peroxygen based disinfectants seems to be effective to inactivate ISAV when using doses recommended by manufacturers. The presence of mucus and blood may, however, reduce the effectiveness of iodophores and chlorhexidine.

ISAV and VNNV are reported to be sensitive to UV exposure.

Ozone treatment of marine fish eggs (halibut, turbot) has been reported to reduce the risk of virus transmission of nodavirus to hatching larvae. Neither ozone nor UV treatment has been tested on salmonid eggs as regards *R. salmoninarum*.

Papers focusing on disinfection procedures for *F. psychrophilum* are reported not to exist although some papers have reported on susceptibility of other *Flavobacterium* species to bactericidals. Similarly, there is little information as regards the susceptibility of *P. salmonis* to common disinfection procedures.

Protocols for egg disinfection

Regarding egg disinfection, this is practiced in different ways from country to country. Iodophor-based disinfectants are dominating, but disinfection procedures varies in key factors like contact time and at what egg development stage treatment is initiated.

Several countries have protocols for disinfection of eggs of salmonids, but protocols for the disinfection of eggs of marine fish species seem to be more or less absent. Iodophores are used on eggs both from salmonid species and marine species, but the scientific basis for these routines are scarce, especially for non-salmonid species. In order to give scientifically based recommendations, there is a need for extensive research effort on topics like disinfectant efficiency, egg toxicity and teratogenicity. A uniform disinfection protocol is not realistic; instead species specific disinfection protocols should be the aim.

Disinfection of eggs has biological relevance in national as well as international trade as regards prevention against disease agents. However, in many countries disinfection of eggs are carried out and some countries seem to have compulsory disinfection.

Chile, Denmark, England/Wales, Italy, Norway, Scotland, Sweden, Tasmania and USA have established a policy and protocols for disinfection of salmonid eggs but while Norway has a protocol for disinfection for both the green egg stage and the eyed egg stage, Chile,

Denmark, England/Wales, Italy, Sweden and USA have no mandatory or generally practised disinfection of newly fertilised eggs. This is however, also recognised as prudent fish hatchery technique in Canada in operations where IHNV have been detected. Ireland has no written instructions in written, but use of an iodine disinfectant is recommended.

Furthermore, disinfection practise in newly fertilized salmonid eggs differs as to timing in relation to egg activation (hardening). In some countries the eggs are disinfected prior to activation, in some during activation and in others after activation. These differences can influence disinfection effect. During activation the egg surface changes considerably (Ahne et al., 1985). When disinfecting during egg activation, the disinfectant penetrates the chorium into the perivitelline space. This is done in the USA and Italy, to improve the effect against IHN virus. Disinfection of newly fertilized has a minor negative effect as to egg survival, partly dependent when it is done in relation to egg activation.

In Asia fish egg trade is not a big issue or it does not take place at all. However, in the Philippines iodine disinfection of grouper eggs has been reported. Similarly, Japan is reported to use iodine or ozone disinfection to disinfect eggs.

Although FAO and OIE have established recommendations as regards procedures for disinfection of fish eggs, more work should be carried out in order to establish commonly accepted, detailed protocols for disinfection of eggs of fish species that are traded in order to reduce the possibility for spread disease agent by trade within countries or internationally.

Future research needs

Pathogen survival

There is a need to develop exact knowledge on pathogen survival and inactivation by environmental parameters such as:

- temperature (freezing/thawing, elevation of temperature),
- pH,
- UV (dose studies)
- Ozone (dose studies).

Disinfection

- Assess susceptibility of pathogens to different physical and chemical parameters under field conditions to establish protocols for inactivation of pathogens for disinfection purposes.
- Systematic testing of environmental desirable disinfectants for egg disinfection purposes.
- Studies of disinfectants on eggs of fish species that are traded internationally:
  - Inactivation
  - Toxicity
  - Teratogenicity.
- Transmission experiments using infected gametes (eggs and sperm) to study survival of a given pathogen in:
  - Disinfected eggs (green egg stage, eyed egg stage)
  - Un-disinfected eggs.
- Study the efficacy of disinfectants under variable environmental conditions in order to achieve optimal conditions for disinfection of fish eggs:
  - Marine water
  - Estuarine water
  - Fresh water.

Protocols for the disinfection of fish eggs

Official regulations and practise of egg disinfection prior to trade of fish eggs nationally and internationally differ much between countries. Due to this, it will be of importance to:

- Develop science based, detailed protocols for disinfection to be used as a prerequisite in international trade of fish eggs.
- Provide a uniform recommended concentration to be used of a given disinfectant, and a recommended minimum range of disinfection time to be used regardless of water temperature and other environmental conditions.

Acknowledgements

Our gratitude is expressed towards experts that have supplied information on common disinfection procedures in various countries that were included in the annexes of this report.

This work has been generously supported by a financial grant provided by the EU Commission under contract no: **QLK2-CT-2002-01546**, for which we express our gratitude. However, the sole responsibility for the contents of this report rests with the authors, and any opinion expressed herein do not represent the opinion of the European Community. Neither is the European Community responsible for any use that may be made of information, opinions or data appearing in this report.

Refererences

Infectious haematopoietic necrosis (IHN)

Amend DF and Pietsch JP (1972). Virucidal activity of two iodophors to salmonid viruses. *Journal of Fisheries Research Board of Canada* **29**, 61-65.

Amend DF (1975). Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. *Journal of Wildlife Diseases* **11**, 471-478.

Barja JL, Toranzo AE, Lemos ML and Hetrick FM (1983). Influence of water temperature and salinity on the survival of IPN and IHN viruses. *Bulletin of the European Association of Fish Pathologists* **3**, 47-50.

Batts WN, Landolt ML et al. (1991). Inactivation of infectious hematopoietic necrosis virus (IHNV) by low levels of iodine. *Applied and Environmental Microbiology* **57**, 1379-1385.

Burke J and Mulcahy D (1983). Retention of infectious haematopoietic necrosis virus infectivity in fish tissues homogenates and fluids stored at three temperatures. *Journal of Aquatic Animal Health* **6**, 1244-1249.

Engelking HM, Kaufman J, Groberg WJJr. and LaPatra SE (1991). Vertical transmission and covert infection studies of infectious hematopoietic necrosis virus. In: Fryer, JL (ed.), Proceedings from the Second International Symposium on Viruses of Lower Vertebrates. July 29-31, 1991. Oregon State University, Corvallis, Oregon, pp. 253-259.

Goldes SA and Mead SL (1995). Efficacy of iodophor disinfection against egg surface-associated infectious haematopoietic necrosis virus. *Progressive Fish Culturist* **57**, 26-29.

Gosting LH and Gould RW (1981). Thermal inactivation of infectious haematopoietic necrosis and infectious pancreatic necrosis viruses. *Applied and Environmental Microbiology* **41**, 1081-1082.

Inouye K, Ikeya F, Yamazaki T and Hara T (1992). Virucidal activities of various germicides to infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus. Proceedings from the OJI International Symposium on Salmonid Diseases. Hokkaido University Press, Sapporo, Japan. pp. 308-316.

Kamei Y, Yoshmizu M, Ezura Y and Kimura T (1987). Effects of estuarine and marine waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). *Bulletin of Faculty of Fisheries, Hokkaido University* **38**, 271-285.

Kamei Y, Yoshimizu M, Ezura Y and Kimura T (1988a). Effects of environmental waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). *Journal of Applied Icthyology* **4**, 37-47.

Kamei Y, Yoshimizu M, Ezura Y and Kimura T (1988b). Screening of bacteria with antiviral activity from freshwater salmonid hatcheries. *Microbial Immunology* **32**, 67-74.

LaPatra SE and Rohovec WDJS (1987). Capacity of formalin to inactivate IHN virus. *American Fisheries Society Fish Health Section Newsletter* **15** (3), 4.

LaPatra SE, Rohovec JS and Fryer JL (1989). Detection of infectious hematopoietic necrosis virus in fish mucus. *Fish Patholology* **24**, 197-202.

LaPatra SE, Groberg WJ, Rohovec JS and Fryer JL (1989). The delayed appearance of infectious hematopoietic necrosis virus (IHNV) in steelhead trout *(Salmo gairdneri)*. In: Ahne, W. and Krustak, E. (eds): Viruses of Lower Vetebrates. Springer-Verlag, New York, pp 430-441.

LaPatra SE (1990). Infectious hematopoietic necrosis virus (IHNV) transmission studies in Oregon. *American Fisheries Society Fish Health Section Newsletter* **18** (1), 5-6.

LaPatra SE, Troyer R, Shewmaker G, Jones GR and Kurath G (2001a). Understanding aquatic animal virus survival and trafficking and its role in risk assessment. In: Proceedings of the Office International des Epizooties (OIE) International Conference on Risk Analysis in Aquatic Animal Health 8-10 February 2000, Paris (C. Rogers, ed.) OIE, Paris, pp. 251-258.

LaPatra SE, Batts WN, Overturf K, Jones GR, Shewmaker WD and Winton JR (2001b). Negligible risk associated with the movement of processed rainbow trout from an infectious hematopoietic necrosis virus (IHNV) endemic area. *Journal of Fish Diseases* **24**, 399-408.

MeyersTR, Thoma JB, Follett JE and Saft RR (1990). Infectious hematopoietic necrosis virus: trends in prevalence and the fish management approach in Alaskan sockeye salmon culture. *Journal of Aquatic Animal Health* **2**, 85-98.

Mulcahy D, Pascho R and Jenes CK (1983). Detection of infectious hematopoietic necrosis virus in river water and demonstration of waterborne transmission. *Journal of Fish Diseases* **6**, 321-330.

Pietsch JP, Amend DF and Miller C (1977). Survival of infectious hematopoietic necrosis virus held under various conditions. *Journal of Fisheries Research Board of Canada* **34**, 1360-1364.

Sako H and Sorimachi M (1985). Susceptibility of fish pathogenic viruses, bacteria and fungus to ultraviolet irradiation and the disinfectant effect of UV-ozone water sterilizer on the pathogens in water. *Bulletin of the National Research Institute for Aquaculture* **8**, 51-58.

Toranzo AE and Hetrick FM (1982). Comparative stability of two salmonid viruses and poliovirus in fresh, estuarine and marine waters. *Journal of Fish Diseases* **5**, 223-221.

Traxler GS, Roome JR and Kent ML (1993). Transmission of infectious haematopoietic necrosis virus in seawater. *Diseases of Aquatic Organisms* **16**, 111-114.

Traxler GS, Roome JR, Lauda KA and LaPatra SE (1997). The appearance of infectious hematopoietic necrosis virus (IHNV) and neutralizing antibodies in sockeye salmon *(Oncorhynchus mykiss)* during their migration and maturation period. *Diseases of Aquatic Organisms* **28**, 31-38.

Watson SW, Guenther RW and Rucker RR (1954). A virus disease of sockeye salmon: interim report. U.S. Fish and Wildlife Service, Special Scientific Report 138, 1-36.

Wedemeyer GA, Nelson NC and Smith CA (1978). Survival of the salmonid viruses infectious hematopoietic necrosis (IHNV) and infectious pancreatic necrosis (IPNV) in ozonated, chlorinated, and untreated water. *Journal of Fisheries Research Board of Canada* **35**, 875-879.

Winton JR (1991). Recent advances in detection and control of infectious hematopoietic necrosis virus in aquaculture. *Annual Review of Fish Diseases* **2**, 83-93.

Whipple MJ and Rohovec JS (1994). The effect of heat and low pH on selected viral and fish pathogens. *Aquaculture* **123** (3-4) 179-189.

Wolf K (1988). Infectious hematopoietic necrosis. In: K. Wolf (ed): Fish Viruses and Fish Virus Diseases. Cornell University Press, Ithaca, New York. pp. 83-114.

Yamazaki T and Motonishi A (1992). Control of infectious hematopoietic necrosis and infectious pancreatic necrosis in salmonid fish in Japan. In: Kimura, T. (ed.) Proceedings of the Oji International Symposium on Salmonid Diseases. Hokkaido University Press, Sapporo, Japan, pp.103-110.

Yoshimizu M, Takizawa H and Kimura T (1986). UV susceptibility of some fish pathogenic viruses. *Fish Pathology* **21**, 47-52.

Viral haemorrhagic septicaemia (VHS)

Ahne W and Held C (1980). Untersuchungen über die viruzide Wirkung von Actomar K30™ auf fischpathogene Viren. *Zeitschrift für alle Gebiete der Veterinärmedizin* **35**, 308-318.

Ahne W (1982 a). Untersuchungen zur Tenazität der Fischviren. *Fortschritte der Veterinärmedizin* **35**, 305-309.

Ahne W (1982 b). Vergleichende Untersuchung über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zentralblatt für Veterinarmedizin* **29**, 457-476.

Eskildsen UE and Jørgensen PEV (1974). Jodophorer som desinfektionsmiddel i dambrugene. *Ferskvandsfiskeribladet* **72**, 1-3.

Frost JW and Wellhausen S (1974). Studies on the effect of temperature and glycerol on the inactivation of the virus of haemorrhagic septicaemia (VHS) of rainbow trout by use of the microtiter system. *Zentralblatt für Veterinärmedizin B* **21**, 625-631.

Jørgensen PEV (1973). Inactivation of IPN and Egtved virus. *Riv. It. Piscic. Ittiop.* – A. VIII – N. 4.

Jørgensen PEV (1974). A study of viral diseases in Danish rainbow trout. Their diagnosis and control. 1-101. Thesis, The Royal Veterinary- and Agricultural University, Copenhagen.

Kurita J, Iida Y, Nakajima K and Inouye K (2002). Virucidal effects of various disinfectants on viral hemorrhagic septicemia virus (VHSV) isolated from Japanese flounder. *Fish Pathology* **37**, 175-181.

Mori K, Iida H, Nishizawa T, Arimoto M, Nakajima K and Muroga K (2002). Properties of viral haemorrhagic septicaemia virus (VHSV) isolated from Japanese flounder *(Paralichthys olivaceus). Fish Pathology* **37** (4)**,** 169-174.

Pietsch JP, Amend DF and Miller CM (1977). Survival of infectious haematopoietic necrosis virus held under various environmental conditions. *Journal of the Fisheries Research Board of Canada* **34**, 1360-1364.

Øye AK and Rimstad E (2001). Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC iradiation. *Diseases Aquatic Organisms* **48**, 1-5.

Spring viremia in carp (SVC)

Ahne W (1976). Untersuchungen über die Stabilität des karpfenpathogenen Virusstammes 10/3. *Fisch Umwelt* **2** 121-127.

Ahne W and Held C (1980). Untersuchungen über die viruzide Wirkung von Actomar K30™ auf fischpathogene Viren. *Zeitschrift für alle Gebiete der Veterinärmedizin* **35**, 308-318.

Ahne W (1982 a). Untersuchungen zur Tenazität der Fischviren. *Fortschritte der Veterinärmedizin* **35**, 305-309.

Ahne W (1982 b). Vergleichende Untersuchung über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zentralblatt für Veterinarmedizin* **29**, 457-476.

De Kinkelin P and Le Berre M (1974). Rhabdovirus des poissons. II. Propriétés in vitro du virus de la virémie printanière de la carpe. *Annales Microbiologique, (Paris),* **125A**, 113-124.

Kölbl O (1975). Neue Erkenntnisse über die Bauchwassersucht bei Karpfen. *Österreichs Fischerei* **5/6**, 69-72.

Epizootic haematopoietic Necrosis (EHN)

Langdon JS (1989). Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. *Journal of Fish Diseases* **12**, 295-310.

Infectious salmon anaemia (ISA)

Anonymous (2000). Final Report of the Joint Government/industry Working Group on Infectious Salmon Anaemia (ISA) in Scotland. Published by Scottish Executive, © Crown copyright 2000, ISBN 0 7480 8950 0, 142 pp.

Anonymous (2001). Efficacy of iodophor disinfectant against ISAV (contract Number 2086-110008-001-H86). Final report prepared for New Brunswick Department of Agriculture, Fisheries and Aquaculture, 107 Mount Pleasant Road, St. George, New Brunswick E5C 3K5. By Molecular Group RPC, 921 College Hill Road, Fredericton, New Brunswick E3B 6Z9, 9 pp.

Antec International Ltd (2003). Vircon S efficacy against specific fish pathogens. http:/www.antecint.co.uk/main/virkaqua.htm

AVC Inc. (Subsidiary of the University of Prince Ewards Island), 2002. *In vitro* determination of the disinfectant efficacy of Virkon S against Infectious Salmon Anemia Virus (ISAV). Letter to Antec International, Suffolk, UK.

Falk K, Namork E, Rimstad E, Mjaaland S and Dannevig BH (1997). Characterization of infectious salmon anaemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar L.*). Journal of Virology **71** (12), 9016-9023.

FRS Marine Laboratory Aberdeen (2000). Field Guide, Disinfection Procedures with regards to ISA virus. Version II, January 2000. Ed. Tim Fraser, Marine Laboratory, 23 pp.

Krogsrud J, Falk K, Dannevig BH, Reite OB and Hektoen H (1991). Infeksiøs lakseanemi (ILA). Raport om inaktivering av ILA-agens. Internal report, National Veterinary Institute Oslo, 16 pp.

Smail DA, Grant R, Bain N and Hastings T (2001). Virucidal effect of iodophors, chloramine T and chorine dioxide against cultured infectious salmon anaemia (ISA) virus. Abstract, 10th International Conference of the EAFP, Dublin, Irland, Sept., 0-018.

Torgersen Y (1998). Physical and chemical inactivation of the infectious salmon anaemia (ISA) virus. Proceedings from the 6th annual New England farmed fish health workshop, Eastport, Maine, USA, pp 44-53.

Øye AK and Rimstad E (2001). Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. *Diseases of Aquatic Organisms* **48**, 1-5.

Viral nervous necrosis (VNN)

Arimoto M, Sato J, Maruyama K, Mimura G and Furusawa I (1996). Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV). *Aquaculture* **143**,15-22.

Borghesan F, Beretta P, Maltese C, Ormelli S and Bovo G. (2002). Inactivation of the causal agent of viral encephalo-retinopathy by exposure to ozone: preliminary tests. *Boll. Soc. It. Patol. Ittica* **35**, 24-33.

Frerichs GN, Tweedie A, Starkey WG and Richards RH (2000). Temperature, pH and electrolyte sensitivity and heat, UV and disinfectant inactivation of sea bass (*Dicentrarchus labrax*) neuropathy nodavirus. *Aquaculture* **185**, 13-24.

Grotmol S and Totland GK (2000). Surface disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with ozonated sea-water inactivates nodavirus and increases survival of the larvae. *Diseases of Aquatic Organisms* **39**, 89-96.

Maltese C and Bovo G (2001). Effects of some chemico-phisical treatments on the virus causing the Viral Encephalo-Retinopathy in farmed sea bass (*Dicentrarchus labrax*). *Boll. Soc. It. Patol. Ittica* **31**, 3-16.

Peducasse S, Castric J and Baudin Laurencin F (1999). Abstract book, 9th international Conference¨Diseases of Fish and Shellfish¨, Rhodes,19-24 September 1999.

Infectious pancreatic necrosis (IPN)

Ahne W (1982). Vergleichende Untersuchungen über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zentralblatt für Veterinarmedizin B* **29**, 457-476.

Ahne W, Kelly RK and Schlotfeldt H-J. (1989). Factors affecting the transmission and outbreak of infectious pancreatic necrosis (IPN). In: Lillelund K and Rosenthal, H (eds.) Fish Health Protection Strategies, BMFT, Bonn, pp 19-67.

Bullock GL, Rucker RR, Amend DH, Wold K and Stuckey HM (1976). Infectious pancreatic necrosis: transmission with iodine-treated and nontreated eggs of brook trout (*Salvelinus fontinalis*). *Journal of the Fisheries Research Board of Canada* **33**, 1197-1198.

Desautels D and MacKelvie RM (1975). Practical aspects of survival and destruction of infectious pancreatic necrosis virus. *Journal of the Fisheries Research Board of Canada* **32**, 523-531.

Dorson M and Torchy (1985). Experimental transmission of infectious pancreatic necrosis virus via the sexual products. In: A.E. Ellis (ed); *Fish and Shellfish Pathology*, pp. 251-261.

Dorson M, Rault P, Haffray P and Torhy C (1997). Water-hardening rainbow trout eggs in the presence of an iodophor fails to prevent the experimental egg transmission of infectious pancreatic necrosis virus. *Bulletin of the European Association of Fish Pathologists* **17** (1), 13-16.

Dorson M, Torhy C, Billard R, Saudrais C, Maisse G, Haffray P and Hollebecq MG (1996). Infectious pancreatic necrosis of salmonids: assessment of methods aiming at cutting off egg-associated transmission. *Bulletin Francais de la Peche et de la Pisciculture* **340**, 1-14 (in French).

Elliott DG and Amend DF (1978). Efficacy of certain disinfectants against infectious necrosis virus. *Journal of Fish Biology* **12**, 277-286.

Inouye K Ikeya F, Yamazak T and Hara T (1990). Virucidal activities of various germicides to infectious pancreatic necrosis virus (IPNV). *Fish Pathology* **25** (2), 81-86.

Itoh S, Yoshimizu M and Ezura Y (1997). Disinfectant effects of low level of total residual oxidants in artificial seawater on fish pathogenic microorganisms. *Nippon Suisan Gakkaishi* **63**, (1), 97-102.

Kamei Y, Yoshimizu M, Ezura Y and Kimura T (1987). Effects of estuarine and marine waters on the infectivities of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV). *Bulletin of the Faculty of Fisheries, Hokkaido University* **38**, 271-285.

Liltved H, Hektoen H and Efraimsen H (1995). Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. *Aquaculture England* **14** (2), 107-122.

MacKelvie RM and Desautels D (1975). Fish viruses: survival and inactivation of infectious pancreatic necrosis virus. *Journal of the Fisheries Research Board of Canada* **32**, 1267-1273.

Maisse G, Dorson M and Torchy C (1980). Ultraviolet inactivation of two pathogenic salmonid viruses (IPN virus and VHS virus). *Bulletin Francaise de Pisciculture* **278**, 34-40.

Moewus-Kobb L (1965). Studies with IPN virus in marine hosts. *Annual of The New York Acadademy of Science* **126**, 328-342

Sako H and Sorimachi M (1985). Susceptibility of fish pathogenic viruses, bacteria and fungus to ultraviolet irradiation and the disinfectant effect of UV-ozone water sterilizer on the pathogens in water. *Bulletin of the National Research Institute of Aquaculture* **8**, 51-58.

Smail DA, Huntly PJ and Munro ALS (1993). Fate of four fish pathogens after exposure to fish silage containing fish farm mortalities and conditions for the inactivation of infectious pancreatic necrosis virus. *Aquaculture* **113** (3), 173-181.

Toranzo AE and Hetrick FM (1982). Comparative stability of two salmonid viruses and poliovirus in fresh, estuarine and marine waters. *Journal of Fish Diseases* **5**, 223-231.

Toranzo AE, Barja JL, Lemos ML and Hetrick FM (1983). Stability of infectious pancreatic necrosis virus (IPNV) in untreated, filtered and autoclaved estuarine water. *Bulletin of the European Association of Fish Pathologists* **3**, 51-53.

Tu K-C, Spendlove RS and Goede RW (1975). Effect of temperature on survival and growth of infectious pancreatic necrosis virus. *Infection and Immunity* **11** (6), 1409-1412.

Vestergård Jørgensen PEV (1973). Inactivation of IPN and Egtved virus. *Rivista Italiano Piscicoltura Ittiopatologia* **8**, 107-108.

Wedemeyer GA, Nelson NC and Smith CA (1978). Survival of the salmonid viruses infectious hematopoietic necrosis (IHNV) and infectious pancreatic necrosis (IPNV) in ozonated, chlorinated, and untreated waters. *Journal of the Fisheries Research Board of Canada* **35** (6), 875-879.

Whipple MJ and Rohovec JS (1994). The effect of heat and low pH on selected viral and fish pathogens. *Aquaculture* **123** (3-4), 179-189.

Yoshimizu M, Takizawa H and Kimura T (1986). UV susceptibility of some fish pathogenic viruses. *Fish Pathology* **21**, 47-52.

Øye AK and Rimstad E (2001). Inactivation of infectious salmon anaemia virus, viral haemorrhagic septicaemia virus and infectious pancreatic necrosis virus in water using UVC irradiation. *Diseases of Aquatic Organisms* **48** (1), 1-5.

Bacterial Kidney Disease *(Renibacterium salmoninarum)*

Armstrong RD, Evelyn TPT, Martin SW, Dorward WJ and Ferguson HW (1989). Erythromycin levels within eggs and alevins derived from spawning broodstock chinook salmon *Oncorhynchus tshawytscha* injected with the drug. *Diseases of Aquatic Organisms* **6**, 33-36.

Austin B, Rayment JN (1985). Epizootiology of *Renibacterium salmoninarum*, the causal agent of bacterial kidney disease in salmonid fish. *Journal of Fish Diseases* **8**, 505-509.

Balfry SK, Albright LJ and Evelyn TPT (1996). Horizontal transfer of *Renibacterium salmoninarum* among farmed salmonids via the fecal-oral route. *Diseases of Aquatic Organisms* **25**, 63-69.

Bullock GL, Stuckey HM and Mulcahy D (1978). Corynebacterial kidney disease: egg transmission following iodophore disinfection. *Fish Health News* **7**, 51-52.

Elliott DG, Pascho RJ and Bullock GL (1989). Developments in the control of bacterial kidney disease of salmonid fishes. *Diseases of Aquatic Organisms* **6**, 201-215.

Evelyn TPT, Ketcheson DA and Prosperi-Porta L (1984). Further evidence for the presence of *Renibacterium salmoninarum* in salmonid eggs and for the failure of povidone-iodine to reduce the intra-ovum infection rate in water-hardened eggs. *Journal of Fish Diseases* **7**, 173-182.

Evelyn TPT, Prosperi-Porta L and Ketcheson DA (1986). Persistence of the kidney disease bacterium, *Renibacterium salmoninarum*, in coho salmon, *Oncorhynchus kisutch* (Walbaum), eggs treated during and after water-hardening with povidone-iodine. *Journal of Fish Diseases* **9**, 461-464.

Evelyn TPT (1993). Bacterial kidney disease - BKD. In: Inglis, V., Roberts, R.J., Bromage, N.R. (Eds.), Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, pp. 177-195.

Gaikowski MP, Rach JJ, Olson JJ and Ramsay RT (1998). Toxicity of hydrogen peroxide treatments to rainbow trout eggs. *Journal of Aquatic Animal Health* **10**, 241-251.

Grotmol S, Dahl-Paulsen E and Totland GK (2003). Hatchability of eggs from Atlantic cod, turbot and Atlantic halibut after disinfection with ozonated seawater. *Aquaculture* **221**, 245-254.

Hirvelä-Koski V (2004). *Renibacterium salmoninarum:* effect of hypochlorite treatment, and survival in water. *Diseases of Aquatic Animals* **59**, 27-33.

Pascho R, Landolt M and Ongerth JE (1995). Inactivation of *Renibacterium salmoninarum* by free chlorine. *Aquaculture* **131**, 165-175.

Pascho RJ and Ongerth JE (2000). Method for flow cytometric monitoring of *Renibacterium salmoninarum* inactivation. *Diseases of Aquatic Organisms* **41**, 181-193.

Rach JJ, Schreier TM, Howe GE and Redman SD (1997). Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. *The Progressive Fish-Culturist* **59**, 41-46.

Ross AJ and Smith CA (1972). Effect of two iodophors on bacterial and fungal fish pathogens. *Journal of the Fisheries Research Board of Canada* **29**, 1359-1361.

Schreier TM, Rach JJ and Howe GE (1996). Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout. *Aquaculture* **140**, 323-331.

Sugita H, Asai T, Hayashi K, Mitsuya T, Amanuma C, Maruyama C and Deguchi Y (1992). Application of ozone disinfection to remove *Enterococcus seriolicida, Pasteurella piscicida*, and *Vibrio anguillarum* from seawater. *Applied and Environmental Microbiology* **58**, 4073-4075.

Waterstrat PR and Marking LL (1995). Clinical evaluation of formalin, hydrogen peroxide, and sodium chloride for the treatment of *Saprolegnia parasitica* on fall chinook salmon eggs. *The Progressive Fish-Culturist* **57**, 287-291.

Flavobacterium psychrophilum infection

Amend DF (1974). Comparative toxicity of two iodophors to rainbow trout eggs. *Transactions of the American Fisheries Society* **103**, 73-78.

Brown LL, Cow WT and Levine RP (1997). Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Diseases of Aquatic Organisms* **29**, 213-218.

Dorson M and Michel C (1987). An evaluation of the activity of five quaternary ammonium compounds on main viruses and bacteria pathogenic for salmonids. *Bulletin Français de Pêche et de Pisciculture* **308,** 61-66.

Holt RA (1972). Characterization and control of *Cytophaga psychrophila* (Borg) the causative agent of low temperature disease in young coho salmon (*Oncorhynchus kisutch*). Thesis, Oregon State University, Corvallis.

Kondo M, Kawai K, Kurohara K and Oshima S (2002). Adherence of *Flavobacterium psychrophilum* on the body surface of the ayu *Plecoglossus altivelis. Microbes and Infection* **4**, 279-283.

Kumagai A and Takahashi K (1997). Imported eggs responsible for the outbreaks of cold-water disease among cultured coho salmon in Japan. *Fish Pathology* **32**, 231-232.

Kumagai A, Takahashi K, Yamaoka S and Wakabayashi H (1998). Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila. Fish Pathology* **33**, 123-128.

Madetoja J, Nystedt S and Wiklund P (2003). Survival and virulence of *Flavobacterium psychrophilum* in water microcosms. *FEMS Microbiology Ecology* **43**, 217-223.

Michel C, Antonio D and Hedrick RP (1999). Production of viable cultures of *Flavobacterium psychrophilum*: approach and control. *Research in Microbiology* **150**, 351-358.

Rach JJ, Gaikowski MP and Ramsay RT (2000). Efficacy of hydrogen peroxide to control mortalities associated with bacterial gill disease infections on hatchery-reared salmonids. *Journal of Aquatic Animal Health* **12**, 119-127.

Rucker RR (1948). New compounds for the control of bacterial gill disease. *The Progressive Fish Culturist* **10**, 19-22.

Rucker RR, Johnson HE and Ordal EJ (1949). An investigation on the bactericidal action and fish toxicity of two homologous series of quaternary ammonium compounds. *Journal of Bacteriology* **57**, 225-234.

Schachte JH (1983). Coldwater disease. In: Meyer, FP, Warren JW, Carey TG (eds.): A guide to integrated fish health management in the Great Lakes Basin. Special Publication 63-2, Great Lakes Fishery Commission, Ann Arbor, Michigan, pp. 193-197.

Vatsos IN, Thompson KD and Adams A (2001). Adhesion of the fish pathogen *Flavobacterium psychrophilum* to unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) and n-hexadecane. *Letters in Applied Microbiology* **22**, 178-182.

Vatsos IN, Thompson KD and Adams A (2003). Starvation of *Flavobacterium psychrophilum* in broth, stream water and distilled water. *Diseases of Aquatic Organisms* **56**, 115-126.

Wood JW (1968). Diseases of Pacific salmon: their prevention and treatment. State of Washington, Department of Fisheries, Hatchery Division.

Wood JW (1974). Bacterial gill disease. Columnaris. Low temperature disease. *In*: Diseases of Pacific salmon: their prevention and treatments. State of Washington, Department of Fisheries, Hatchery Division, Olympia, Washington, pp. 18-24.

Piscirickettsiosis *(Piscirickettsia salmonis)*

Lannan CN, Bartholomew J and Fryer JL (1999). Rickettsial and Chlamydial infections. Chapter 6 in: Woo PTK and Bruno DW (eds.): Fish Diseases and Disorders Vol. 3; viral, bacterial and fungal infections, pp. 245-267.

Lannan CN and Fryer JL (1994). Extracellular survival of *Piscirickettsia salmonis. Journal of Fish Diseases* **17**, 545-548.

Larenas JJ, Bartholomew J, Troncoso O, Ledezma H, Fernandez S, Sandoval N, Vera P, Contreras J and Smith P A (2003). Experimental vertical transmission of *Piscirickettsia salmonis* and *in vitro* study of the attachment and mode of entrance into the ovum. *Diseases of Aquatic Organisms,* **56**, 25-30.

Mauel MJ and Fryer JL (2001). Amplification of a *Piscirickettsia salmonis*-like 16S rDNA product from bacterioplankton DNA collected from Oregon, USA coastal waters. *Journal of Aquatic Animal Health* **13**, 280-284.

Mauel MJ and Miller DL (2002). Piscirickettsiosis and piscirickettsiosis-like infections in fish: a review. V*eterinary Microbiology* **87**, 279-289.

Palm RC, Powell DB and Peña MP (1998). *Piscirickettsia salmonis* inactivation by chlorine in fresh water. Proceedings from the International Symposium on Aquatic Animal Health, Baltimore, Maryland, USA. 11, 4.

Smith PA, Pizarro P, Ojeda P, Contreras J, Oyandel S and Larenas J (1999). Routes of entry of *Piscirickettsia salmonis* in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **37**, 165-172.

Annex 1
Chile
– Egg disinfection procedures

MINISTRY OF ECONOMICS
PROMOTION AND
RECONSTRUCTION
NATIONAL FISHERIES SERVICE

APPROVING THE GENERAL SANITARY PROGRAM FOR THE DISINFECTION OF SALMONID EGGS (PSGO)

VALPARAISO, JANUARY 24, 2003
No 65 / HAVING READ: the report by the Technical Committee; the provisions set forth in DFL No5, of 1983 and its modifications; D.S. N°430 of 1991, that sets the amended, coordinated and systematized purview of Law No18.892 and its modifications, Fisheries and Aquaculture Act; D.S. No319 of 2001, all issued by the Ministry of Economics, development and Reconstruction; and in Resolution No520 of 1996 issued by the National Comptrollership of the Republic.

CONSIDERING:
That the Regulations for Protection, Control and Stamping Out Measures of High Risk Diseases for Hydro biological Species, approved by D.S. No319 of 2001, issued by the ministry of Economics, Promotion and Reconstruction has entrusted the National Fisheries Service with the establishment of general and specific sanitary programs applicable to all activities covered by those Regulations. That the adequate operational sanitary measures shall be determined by the general sanitary programs, according to each hydro biological species used or farmed, thus promoting proper health conditions for said species and preventing the spreading of diseases.

RESOLVES:
TO APPROVE the following General Sanitary Program for the Disinfection of Salmonid Eggs (PSGO):

GENERAL SANITARY PROGRAM DISINFECTION OF EGGS

I. PURPOSE OF THE PROGRAM
The purpose of this program is to establish the sanitary procedures applicable to the disinfection of eggs, aiming at preventing the spreading of eventual pathogen agents.

II. SCOPE
This Program shall apply to the production of national and imported eggs of salmonid species.

III. DEFINITIONS
The following definitions shall apply to this Program:

1. Egg: fertilized and viable ovule of an aquatic animal.
2. Embryonated egg: eggs of fishes in which the eyes of the embryo are visible. They are also known as eggs with eyes.
3. Batch of eggs: group of eggs in a farm belonging to the same species, proceeding from the same spawning and that have always shared the same water supply.

IV. GENERAL ASPECTS

1. Farms where eggs are hatched shall keep a manual describing the procedures for disinfection and those responsible for this activity.
2. Farms must have a records system in place in order to document the disinfection procedures applied to each of the egg batches entering the hatching system.

V. PROCEDURES

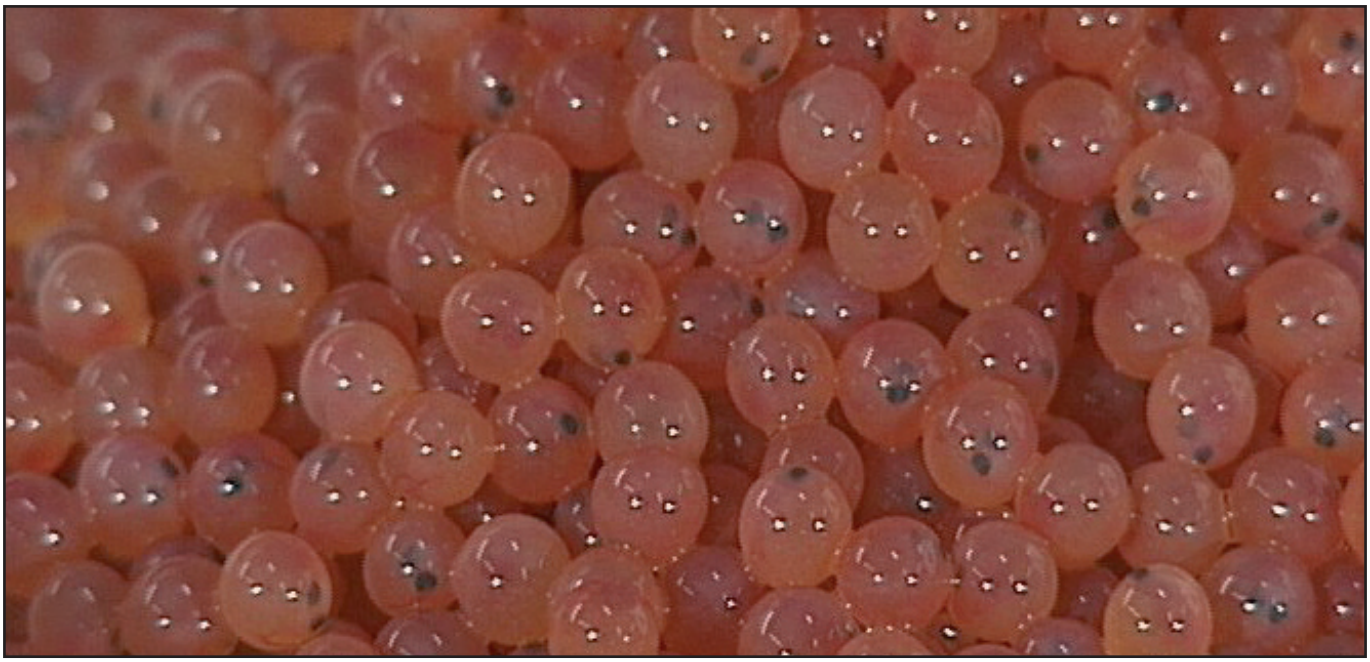
1. The surface of the embryonated eggs of salmonid species shall be disinfected in the hatchery of destination and subsequently be placed in running water free of pathogen agents.
2. Disinfection shall be conducted using the following procedure:
    a. Prepare an iodized solution using clean water. This solution should constantly contain at least 100 ppm of free iodine.
    b. Control the pH of the iodized solution, keeping it between 6 y 8.
    c. Submerge the eggs in the iodized solution during 10 minutes. Application shall not exceed 2000 eggs per liter of disinfectant solution. Use an abundant amount of solution replacing it when it acquires a light-yellow tone and before color disappears.

d. Eggs must be rinsed with abundant water before and after disinfection. The resulting liquid residues must be disposed of in a manner non-detrimental to the environment.
e. The packaging used during transportation of the eggs must also be disinfected, or better yet, destroyed through a procedure that prevents any sanitary hazard or contamination in the water and/or other fishes of the place of destination.
f. Farms where hatching takes place must notify the nearest Service office on the date and time of disinfection with at least 24 hours in advance.

ARTICLE TWO:
FOR REGISTRATION, TRANSMISSION AND PUBLICATION.

SERGIO MUJICA MONTES NATIONAL DIRECTOR OF FISHERIES DISTRIBUTION

- Department of Fisheries Health
- Legal Department
- Office of Reports



Annex 2
OIE guidelines for disinfection of fish eggs

Article 5.2.1.1: Introduction

Although generally effective for decontamination of surfaces of eyed and newly fertilised eggs, the use of *disinfectants*, such as iodophors, cannot be relied upon to prevent vertical transmission of some bacterial (e.g. *Renibacterium salmoninarum*) and viral pathogens (e.g. infectious pancreatic necrosis virus) that may be present within the eyed and newly fertilised egg.

Article 5.2.1.2: Conditions of use

The pH of the solutions of the iodophor products must be between 6 and 8. At a pH of 6 or less, the toxicity for eyed and newly fertilised *eggs* increases, and at 8 or more, the *disinfection* efficacy decreases. It is therefore essential to control the pH, and 100 mg/litre of NaHCO3 must be added to water with a low alkalinity value. It is recommended that the *eggs* be rinsed in fresh water before and after *disinfection*, or that the iodine, after the appropriate contact time, be neutralised with sodium thiosulfate, and that water free from organic matter be used to prepare the iodophor solution. The contact time at the concentration of 1 litre of 100 ppm of iodophor solution should not be less than 10 minutes and the solution should be used only once. Additionally, for sanitising newly fertilised salmonid eggs via a water-hardening process with iodophors, the active ingredients should be no less than 50 ppm, the disinfection period no less than 30 minutes, and the solution should be used only once. For the other species, preliminary tests should be conducted to determine at what egg stage and with what type/concentration of *disinfectant, disinfection* can be carried out.

Finally, in the case of *eggs* that have been transported, the packaging should also be disinfected or, better still, destroyed in a manner that will not pose a contamination or health risk to water and/or other *fish* at the end destination.

Certain precautions must be taken prior to the use of iodophors as products on the market contain a variable quantity of detergents that can give rise to toxic effects. It is therefore recommended that preliminary tests be carried out among the products on the market. It is advisable to build up stocks of the most satisfactory product, but expiry dates must be considered.

Disinfection of *eggs* with iodine can be carried out for the various *fish* species but it is most commonly used for *fish* of the Salmonidae family. For the other species, preliminary tests should be conducted to determine at what egg stage and iodophor concentration disinfection can be carried out safely. *Disinfection* of *eggs* of marine species, such as plaice, cod, Atlantic halibut, for which adverse effects have been documented, may be obtained with 400–600 mg/litre glutaraldehyde with a contact time of 5–10 minutes. However, this is not effective against nodaviruses, for which the use of ozone at 1 mg O^3/litre for 30 seconds is recommended. A concentration of ozone of 0.1–0.2 mg O^3/litre for 3 minutes inactivates most pathogenic fish bacteria as well.

Article 5.2.1.3: Efficacy limits

Disinfection of eggs with iodine is ineffective when trying to avoid vertical transmission of infectious pancreatic necrosis, renibacteriosis and even infectious haematopoietic necrosis, for which this method was recommended initially. The ineffectiveness of iodine has been proved by epidemiological surveys and laboratory tests.

Annex 3

England and Wales regulation for disinfection of fish eggs upon import (DOF 4)

1. Procedures

The ova should be placed for treatment in a suitable container such as an enamel bowl, plastic or fibreglass tray, or a hatching tray. The choice of container will depend to some extent on the number of eggs to be treated. A solution of the iodine disinfectant "Wescodyne" at a strength of 50 ppm in water should be poured on the eggs until they are well submerged. Alternatively, the container and ova can be completely immersed together in a larger volume of appropriately diluted disinfectant held in a treatment bath.

The ova should be left in contact with the disinfectant for 10 minutes and given gentle agitation from time to time to ensure good contact between the disinfectant and the surface of the ova. After the 10 minute treatment period, the ova must be rinsed thoroughly several times in clean water before they are placed in their hatching trays.

Immediately after the ova have been taken from their transport packaging and containers, all packaging and containers must either (a) be destroyed by burning; or (b) be disinfected using chlorine– or iodine-based disinfectant at the manufacturers recommended concentration and disposed of to a landfill facility.

Immediately after the ova have been taken from their transport packaging and containers, all packaging and containers must either (a) be destroyed by burning; or (b) be disinfected using chlorine– or iodine-based disinfectant at the manufacturers recommended concentration and disposed of to a landfill facility.

Transport packaging and containers must not be recycled or reused. Wherever possible, the whole process of unpacking and disinfecting the ova should be completed away from the hatchery. It is a dangerous practice to take ova obtained from outside sources into a hatchery before they have been completely disinfected. Any equipment which has been in contact with the ova before treatment should also be disinfected with the Wescodyne solution, or burnt. Do not forget that handling infected eggs will leave infection on the hands or overalls. Wash hands in disinfectant and send overalls to laundry.

The disinfectant possesses a built-in colour indicator of its own activity and germicidal action continues as long as a yellow/amber colour remains. Colourless or very pale yellow solutions are inactive and should not be used.

Wescodyne can be obtained from several chemical suppliers in the UK. The name and address of the nearest supplier can be obtained from CIBA Agrochemicals, Whittlesford, Cambridge, CB2 4QT. It is usually supplied as a concentrated solution to be diluted at the rate of 1 fluid ounce per 2 gallons of water to give the working solution of 50 ppm available iodine. In areas with soft and acidic water, where the pH is less than 6.5 (or where the pH is not known) 1 teaspoonful of sodium bicarbonate (baking soda) should be added to each 2 gallons of solution to ensure that the pH remain at or above this value. Buffodine™ (Evans Vanodyne, Bamber Bridge, Preston Lancs) may be used as an alternative to Wescodyne and should not be adjusted to pH since it is already buffered.

2. Precautions

At a strength of 50 ppm available iodine, Wescodyne is relatively non-toxic to fertilised trout ova and, with an exposure time of 10 minutes and a pH above 6.5, there is a considerable margin of safety. However, at the strength recommended **the solution is highly toxic to unfertilised ova (green eggs) and to live fish**. Great care must be exercised therefore, whenever Wescodyne is used in a hatchery containing fry.

All Wescodyne solutions and washing should bepreferably be disposed of into a sewer, but where this is not possible they can be safely released very slowly into fast-flowing water.

Annex 4

Italy - Egg disinfection procedures (modified after La Patra SE)

1)
Spawn eggs into colander and separate from ovarian fluid. This step removes ovarian fluid which can contain high concentrations of bacteria and virus that can contaminate eggs and sperm. The ovarian fluid also contains protein and other organics which can reduce the disinfectant concentration of the iodophor solution decreasing its effectiveness.

2)
Rinse eggs with 0.9% saline (30-60 seconds). This step further removes microorganisms and organics which may be loosely bound to the egg surface.

3)
Add sperm and fertilize for 5-15 minutes. Fertilization times can vary (2-10 min). Wet or dry fertilization are acceptable.

4)
Rinse in 0.9% saline (30-60 seconds). This step removes excess sperm and other organic materials.

5)
Rinse in 100 ppm iodophor solution (1 min). During this brief iodophor exposure, iodine will combine rapidly with any remaining organics resulting in a decline in the disinfectant activity. The volume of iodophor used should just cover the eggs and be discarded after the 1 minute rinse.

6)
Disinfect eggs for 15-30 minutes. The iodophor solution used in this step should retain full activity during the disinfection / water-hardening process because of the pretreatment in step #5. Iodophor should be used during the first period of water-hardening so the iodophor is drawn into the perivitelline space of the egg. The ratio of eggs to iodophor solution should be a minimum of 1:4. Recirculation of the iodophor solution can be done during the disinfection period to evenly distribute the active iodine.

7)
Rinse iodophor from eggs using clean or sterilized hatchery water (30-60 seconds). Clean or sterile hatchery water should be used. Eggs will continue to water-harden for approximately 90 minutes and bacteria, virus(es), or other contaminants could be drawn into the perivitelline space of the egg during this period if contaminated water is used.

8) Finish water-hardening in clean water. Use clean or sterile hatchery water. Make sure water has adequate oxygen, pH, etc.

Annex 5

Norway - Standard procedure disinfection of newly fertilized eggs

(Unauthorised translation of the Manual of Instructions used in Norwegian wild fish cultivation hatcheries):

1. Purpose

To reduce the risk of transmission of contagious disease from brood fish to the next generation of fish.

2. Application

The procedure must be used for all disinfection of newly fertilized salmonid eggs in hatcheries affiliated to the "Health surveillance in salmonid fish" project.

3. Responsibilities

The site manager has the responsibility to see that all newly fertilized eggs to be further used in production must be disinfected in accordance with this procedure.

The responsible professional person in charge has the responsibility for the technical control of this work.

4. Necessary equipment

1.Reagents:

a. Disinfectant for eggs (Buffodine™)
b. Ordinary kitchen salt
c. Pathogen free water

2. Equipment

a. Buckets/basins/bowls, possibly with lid
b. Container for physiological salt water solution
c. Disposable spoons or similar
d. Tanks/hatching trays
f. Salinity measuring equipment e.g. refractometer

5. Performance

5.1 Pre-treatment

5.1.1. When the eggs have been stripped, they must be rinsed a minimum of twice in a physiological salt solution or another similar sterile buffer solution prior to fertilization.

NB! Ensure that the saltwater solution is not below 0.9 %. Lower concentrations may cause swelling of the eggs.

5.1.2. Add milt – stirring

NB. Use only new disposable spoons – a new spoon for each batch of eggs, hatching cylinder or hatching tray.

5.1.3. After fertilization, the eggs must be rinsed a minimum of once in physiological salt water or in another sterile buffer solution.

5.2 Disinfection

5.2.1. Disinfection of the eggs must take place as soon as possible after the last rinsing step and prior to swelling.

5.2.2. For disinfection the following preparation shall be used: Iodophor solution giving 100 ppm free iodine in physiological salt water solution (0.9 % NaCl).

When using the commercial product Buffodine™, this corresponds to the following proportion of mixture:

- Buffodine™: 1 dl
- Ordinary table salt: 90 g
- Pure water: 10 l

5.2.3. Disinfection time: Minimum 10 minutes.

5.2.4. The whole bucket/basin/bowl with eggs shall be dipped under the surface of the disinfection bath for the whole disinfection period.

5.2.5. Period of use – ready to use solution: Maximum 1/2 day (1 working period).

5.3. Organisation of the work

5.3.1. The whole process of rinsing and disinfection shall be carried out following a "production line" with adequate space between each operation.

The work must be carried out in such a way that there should be a minimum of crossing to and fro between the different steps in the process.

There must be a distinct physical partition between unclean (prior to disinfection) and clean areas (after disinfection). See flow chart according to specification HO – 1310.

This partition must be constructed in such a way that it prevents person traffic and water pollution, etc. between clean and unclean areas.

Different personnel must be used for simultaneous operation of clean and unclean areas.

If personnel have to carry out work in both areas, strict hygiene precautions must be taken including full change of working clothes, foot wear and hand washing.

STANDARD PROCEDURES DISINFECTION OF EYED EGGS

1. Purpose
To reduce the risk of transmission of contagious disease from brood fish to the next generation of fish.

2. Application
The procedure must be used for all disinfection of newly fertilized salmonid eggs in hatcheries affiliated to the "Health surveillance in salmonid fish" project.

3. Responsibilities
The site manager has the responsibility to see that all newly fertilized eggs to be further used in production must be disinfected in accordance with this procedure.

The responsible professional person in charge has the responsibility for the technical control of this work.

4. Necessary equipment
Ingredients:

1 Disinfectant for eggs (Buffodine™)
2 Ordinary kitchen salt
3 Pathogen free" water

Equipment

1. Buckets/basins/bowls, possibly with lid
2. Container with physiological salt water solution
3. Disposable spoons or similar
4. Tanks/hatching trays
5. Salinity measuring equipment e.g. refractometer

5. Performance
5.1. The disinfection of the eggs must take place immediately prior to packing and transport out of the hatchery or prior to transfer of the eggs to own hatchery/start feeding unit in the hatchery.

5.2. For disinfection the following preparation shall be used:

Iodophor solution giving 100 ppm free iodine in physiological salt water solution (0.9 % NaCl).

When using the commercial product Buffodine™, this corresponds to the following proportion of mixture:

- Buffodine™: 1 dl
- Ordinary table salt: 90 – 100 g
- Pure water: 10 l

5.3. Disinfection time: Minimum 10 minutes.

5.4. The whole bucket/basin/bowl with eggs shall be dipped under the surface of the disinfection bath during the whole disinfection period.

5.5. Period of use – ready to use solution: Maximum 1/2 day (1 working period).

6. Organising of the work
6.1. The whole process of rinsing and disinfection shall be carried out following a "production line" with adequate space between each operation.

The work must be carried out in such a way that there should be as little crossing to and fro between the different steps in the process.

6.2. There must be a distinct physical partition between unclean (prior to disinfection) and clean area (after disinfection). See flow chart according to specification HO – 1310.

This partition must be constructed in such a way that it prevents person traffic and water pollution, etc. between clean and unclean area.

6.3. Different personnel must be used for simultaneous operation of clean and unclean areas.

If personnel have to carry out work in both areas, strict hygiene precautions must be taken including full change of working clothes, foot wear and hand washing.

Annex 6

Sweden

- Procedures for disinfection of eggs

(Unauthorised translation of the Manual of Instructions issued in Älvkarleby 2003 Fact sheet, © 2003 Fiskhälsan FH AB.

Disinfection of fertilised eggs

General

Upon all transportation of fertilised eggs between two different hatcheries, disinfection of the eggs must be carried out. This comes also into force when eggs are moved from brood stock farms to the hatchery or between two different hatcheries or fish farms. Disinfection of eggs should also preferably take place prior stocking into the wild.

The purpose of egg disinfection is to prevent spread of diseases of viral or bacterial origin and is a cheap insurance to avoid introduction of a contagious disease into the farm, into the hatchery or to the wild.

Various iodophor preparations are those that are mostly used in this regard during later years, which by using the recommended concentrations, have lethal effect on both virus and bacteria. The effect of disinfection is obtained by keeping the eggs in the iodophor solution over some time.

Transport boxes, packing material and others following the egg delivery from another hatchery, may be contaminated and must thus immediately be handled in such a manner that contamination is avoided.

Preparations

Examples of commercially available products are Buffodin® and Actomar®K30. Normally, these preparations contain 1 % free iodine and buffered to neutral pH. The shelf life is usually one to two years, and the solution should be black or dark brown.

Iodophor preparations are stable complexes of an organic composition of the type PVP (poly-vinyl-pyrrolidone) and iodine in water solution. The effect of disinfection is due to slow release of iodine from the complex and acts oxidative. Iodophor preparations are neutralised by organic substances and detergents. The preparations have no occupational hazard if the instructions (from the producer) are followed.

Dosage

Dilute the concentrated solution according to the instructions given on the packaging, normally 100 ml to 10 litres of water is used which means 100 ppm iodine when there is 1% free iodine in the concentrated solution.

Add 90 g sodium chloride (salt) to 10 litres of water (i.e. 0,9 % solution) to be used as disinfection on newly fertilised eggs (green eggs). This procedure prevent uptake of water into the eggs if the swelling is not completely ended.

Dip the eggs in the diluted solution for 10 minutes and use maximum 20 000 eggs per 10 litres of the ready for use solution. Wash the eggs several times in clean water prior to delivery.

If the "bathing" (egg disinfection) is carried out in the hatchery where the eggs are to be introduced, the washing may take place in the hatching trays of the receiving hatchery. Eggs must not be transported in the iodophor solution.

Eyed eggs

Eyed eggs may be dipped directly into the diluted iodophor solution, provided that the water in which the eggs are kept and the iodophor solution have the same temperature.

It may also be appropriate first to pick and discard dead eggs. Upon washing of the eggs following disinfection at the hatchery of delivery, water from another source than the water normally used by the hatchery, should be used.

It is not recommended to disinfect the eggs later that one week prior to estimated hatching time.

Newly fertilised eggs

Newly fertilised eggs shall be allowed to finish hardening ("swell") (1-3 hours at 5-10°C) prior to disinfection and use an addition of 0.9% sodium chloride (see above).

Newly fertilised eggs shall be bathed as gentle as possible to avoid mortality. The eggs should thus not be moved between to many containers.

Annex 7

Tasmania - Recommendations for disinfection of fish eggs

K. Ellard: Fish Health Unit Technical Information Sheet no 4.1, May 2003.

General Precautions and Procedures

- 100ppm available iodine for 10 minutes is normally recommended for salmonid eggs.

- It is essential that the pH of the iodophor solution is maintained between 6.0 and 7.5 (preferably 7.0 to 7.5) for the disinfection process to be successful. At a pH 6.0 or less (acidic solutions) the toxicity of the solution to eggs increases, whilst at pH 7.5 or more (alkaline solutions) the disinfectant effect is reduced. It is therefore essential to test the pH of the solution prior to use. As a general rule, povodine-iodine solutions will be neutral and not require buffering, but the solution must still be checked. Solutions may be buffered using 100mg sodium bicarbonate ($NaHCO^3$) per litre of diluted iodophor solution if the pH is low.

- Fish eggs should be treated in a container that is large enough to contain ten times as much diluted iodophor by volume as the volume of eggs to be treated. Clean plastic washing up bowls, buckets or bins are ideal containers for disinfecting moderate quantities of eggs.

- The eggs should be rinsed in clean fresh water before disinfection to remove high levels of organic matter contained in the ova fluid. They should again be rinsed after disinfection to remove traces of the disinfection solution. Proteins rapidly inactivate iodophors, therefore rinsing eggs prior to disinfection in addition to using good quality water will extend the life of the solution.

- Generous amounts of the solution should be used and the solution replaced when it turns yellow or loses colour. One litre of solution at a concentration of 100mg/L will disinfect approximately 2000 salmonid eggs.

- The diluted iodophor solution may be used to disinfect successive batches of eggs provided it does not become exhausted, which is generally indicated by as change in colour. To check on solution exhaustion, keep a whole (white) cup of the original disinfection solution to one side and compare this with the used disinfectant after each successive batch.

- Care should be taken in the disposal of used solutions. Iodine is toxic to fish and should not be emptied into ponds, tanks or natural waterways. Dispose of used solutions away from waterways in pits.

- If iodophors are to be used on newly fertilised eggs they should have finished hardening prior to disinfection otherwise some iodine may be absorbed into the egg and be toxic to the developing embryo.

Suggested Egg Disinfection Protocol

a After hardening, pour eggs into rinse bin with sieve bottom
b Rinse eggs in system water
c Bathe eggs in 100 ppm available iodine (pH 7) solution for 10 mins
d Rinse eggs in system water
e After approximately three batches or when solutions loses colour it should be replaced with a fresh solution
f Lay out eggs into trays or incubators.

Iodophor Solution

- 500ml 'Betadine', 'Minidine' or 'PVP-Iodine Solution' in 50 litres system water to achieve concentration of 100ppm available iodine (based on 1% or 10mg/ml available iodine).

- If necessary, add 5g sodium bicarbonate to solution and mix.

Check that pH is between 7.0 and 7.5.

Annex 8

North American iodophor water-hardening procedure for salmonid eggs (after LaPatra SE)

1)
Spawn eggs into colander and separate from ovarian fluid. This step removes ovarian fluid which can contain high concentrations of bacteria and virus that can contaminate eggs and sperm. The ovarian fluid also contains protein and other organics which can reduce the disinfectant concentration of the iodophor solution decreasing its effectiveness.

2)
Rinse eggs with 0.9% saline (30-60 seconds). This step further removes microorganisms and organics which may be loosely bound to the egg surface.

3)
Add sperm and fertilize for 5-15 minutes. Fertilization times can vary (2-10 min). Wet or dry fertilization are acceptable.

4)
Rinse in 0.9% saline (30-60 seconds). This step removes excess sperm and other organic materials.

5)
Rinse in 100 ppm iodophor solution (1 min). During this brief iodophor exposure, iodine will combine rapidly with any remaining organics resulting in a decline in the disinfectant activity. The volume of iodophor used should just cover the eggs and be discarded after the 1 minute rinse.

6)
Disinfect eggs for 15-30 minutes. The iodophor solution used in this step should retain full activity during the disinfection / water-hardening process because of the pretreatment in step 5). Iodophor should be used during the first period of water-hardening so the iodophor is drawn into the perivitelline space of the egg. The ratio of eggs to iodophor solution should be a minimum of 1:4. Recirculation of the iodophor solution can be done during the disinfection period to evenly distribute the active iodine.

7)
Rinse iodophor from eggs using clean or sterilized hatchery water (30-60 seconds). Clean or sterile hatchery water should be used. Eggs will continue to water-harden for approximately 90 minutes and bacteria, virus(es), or other contaminants could be drawn into the perivitelline space of the egg during this period if contaminated water is used.

8)
Finish water-hardening in clean water. Use clean or sterile hatchery water. Make sure water has adequate oxygen, pH, etc.

Annex 9

FAO manual for seabass and seabream

From: http://www.fao.org/DOCREP/005/X3980E/x3980e00.htm#Contents

Weighing, disinfecting and counting eggs

Prior to stocking eggs, either into the hatching facilities or directly into the larval rearing tanks, three more steps are required: weighing, estimation of their quantity and disinfection.

Even a rough estimate of the egg numbers allows the person responsible of the larval rearing sector to properly plan the stocking of the larval tanks, to optimise production routines and to coordinate the work of related sectors (live feeds and weaning). It does also allow a proper evaluation of the final survival rate to be expected.

The procedure to weigh the eggs consists in dividing them in many sub-samples, taking each of them out of the temporary container in a plastic filter removing quickly the excess of water, and weighing them on a balance calibrated for the egg filter tare. They are immediately returned to the temporary container (or disinfected, see below). During the collection of the eggs for this operation, only floating, viable eggs are picked. Dead and unfertilized eggs are thus discarded twice, a first time in the collector and then on this occasion.

Egg disinfection is the very first effective barrier against transmission of fish diseases, and is therefore highly recommended for all batches of eggs, both those produced in the hatchery and those brought from other hatcheries. This important operation is usually conducted just after the weighing, when the filter containing the egg sub-sample is dipped in the disinfecting bath for a short period of time prior to being put into the incubation tank. The most commonly used egg disinfectants are Penicillin G, Streptomycin sulphate and active iodine. Even if these antibiotics are commonly used, due to the undesirable side-effects they have and the risks to induce bacterial resistance, active iodine is suggested as the preferred disinfectant.

The assessment of egg numbers can be made in two ways: by relating number to weight or by counting. In the first case the total egg weight is divided by the average individual egg weight, assessed from a small sample. The second method contemplates counting the eggs present in a few 1-l sub-samples and multiplying the average value by the total tank volume. While the water taken with the egg samples biases the first method, the latter requires a uniform egg distribution in the tank to be statistically correct. This method can also be applied to count freshly hatched larvae, which gives a better estimate of the initial population.

The protocol to weigh, count and disinfect eggs is given below:

FISH EGGS WEIGHING, DISINFECTING AND COUNTING OPERATIONS

The following equipment is needed to weigh and disinfect eggs:

- a couple of plastic filters with a 500 mm net (a 12-cm high cylinder cut from a 20-cm diameter PVC pipe with plankton nylon net glued to one end) and with its weight (tare) marked on the external side of the filter;
- a few 10 to 15 l plastic buckets or large beakers with handle;
- a couple of plastic jars, of a capacity of one liter each;
- aeration devices for buckets (fine air diffusers, plastic hoses and air taps);
- oxygen supply emergency set (oxygen bottle, manometer, fine diffusers, plastic hoses and air taps);
- glassware to sample mix and distribute the disinfectants (pipettes, spoons, beakers, glass rods, disposable plastic gloves);
- a balance with automatic tare clearance.

Working protocol to be followed when weighing and disinfecting eggs:

1. prepare all the equipment, which should be sterilized and carefully rinsed. Prepare pencil and paper;

2. prepare the containers with sterilized seawater at the same temperature and salinity of the spawning tanks; provide a gentle aeration; prepare one container adding the selected disinfectant (see table below for dosage and use);

3. dip the filter in the egg temporary stocking container and gently scoop out at the most 150-200 g of eggs floating at the water surface;

4. take the filter with eggs out of the water, drain quickly most of the water in excess on a paper filter and weigh the filter with the eggs; record the weight on a form (if the balance has not been adjusted for the filter tare, the filter weight has to be subtracted);

5. quickly place the filter in a bucket containing well aerated seawater with the selected disinfectant (see table below); wait for the proper disinfection time;

6. transfer the eggs to the incubation tank and open the seawater circuit.

Note - *Do not start operations if something is still not ready as time is essential in egg handling to reduce stress and risks.*

Disinfectants used for seabass and gilthead seabream eggs

Active substance	Dosage	Time	Use
Penicillin	80 I.U./ml	1 min	500 mg/10 l of sea water for 100-200 g of eggs at a time
Streptomycin-SO_4	50 mg/ml	1 min	500 mg/10 l of sea water for 100-200 g of eggs at a time
Active Iodine	50 ppm/litre	10 min	8 litres to be used for: 1 x 10^6 seabass eggs or 1.5 x 10^6 gilthead seabream eggs

Working protocol to follow when counting eggs:

1. close the water inlet in the tank and adjust aeration to assure a uniform distribution of eggs;

2. take several samples of water and eggs by means of a volumetric pipette (tip off) or a beaker, sample size between 10 and 100 ml, according to egg density, the bigger the sample, the lesser should be the egg number. A minimum of five samples are required;

3. count the eggs in each sample, obtain the average number per unit of volume, say one liter, then multiply this number by the total water volume to obtain the total amount of eggs in the tank.

Note - *Only officially appointed personnel should handle all chemicals; product choice and dosage must strictly follow national regulations*

Alternative counting system (starting at step 6, see above):

1. fill a few 30-l cylindro-conical tubs with sterilised seawater and provide with gentle aeration;

2. using the 500 µm filter (see above for description) transfer eggs into the counting tubs and adjust water volume to a known value;

3. take at least 5 samples per tub using a 1-ml pipette; count the eggs;

4 after disinfection, transfer to the incubation containers in clean buckets.

WARNING

Avoid overloading the filter when weighing and do not allow eggs to dry or to stick together in a thick layer; the whole weighing process should last a few seconds only. Avoid any unnecessary manipulation. Avoid any mechanical shock to the eggs.