STATE OF MAINE

DEPARTMENT OF ENVIRONMENTAL PROTECTION

BOARD OF ENVIRONMENTAL PROTECTION

	IN THE MATTER OF
NORDIC AQUAFARMS, INC.	:APPLICATIONS FOR AIR EMISSION,
Belfast and Northport	:SITE LOCATION OF DEVELOPMENT,
Waldo County, Maine	:NATURAL RESOURCES PROTECTION
	:ACT, and MAIN POLLUTANT
	:DISCHARGE ELIMINATION SYSTEM
	:(MEPDES)/WASTE DISCHARGE
A-1146-71-A-N	:LICENSE
L-28319-26-A-N	:
L-28319-TG-B-N	:
L-28319-4E-C-N	:
L-28319-L6-D-N	:
L-28319-TW-E-N	:
W-009200-6F-A-N	:
ME0002771	

Assessment of the Nordic Aquafarms Permit to Satisfy Clean Water Act Requirements

TESTIMONY/EXHIBIT: NVC/UPSTREAM 8

TESTIMONY OF: Bill Bryden

DATE: December 13, 2019

Review of Issues and Flaws with the Application Made by Nordic Aquafarms by Bill Bryden B.Sc

Alternatives Analysis :

There are several alternative commercial methodologies that are in use and delivering Atlantic Salmon to market in the United States, Canada and worldwide. They represent best practices for Land Based RAS aquaculture.
These commercial methodologies result in zero effluent discharged into public waters and drastically reduced usage of freshwater input. They include both saltwater grow-out of salmon as well as freshwater grow-out.

In Canada, Sustainable Blue has commercialized a licenseable methodology for zero effluent discharge for Atlantic Salmon in saltwater as well as other species. They recycle 100% of all water they utilize, both fresh and salt except for that water that resides in the fish when slaughtered. This is accomplished

by utilizing a combination of ozone and advanced filtration methodologies. Their fish is marketed and distributed throughout eastern Canada. Sustainable Blue's CEO and Chief Technology Officer has reviewed the public documents describing Nordic's proposed facility in Maine and have confirmed their methodology can scale to accommodate Nordics proposed volumes of fish. They are prepared to do so under license to Nordic.

In the United States, Superior Fresh has commercialized their freshwater operation in Wisconsin. They have utilized greenhouses to receive their freshwater effluent. They are marketing their salmon and greenhouse produce. While Nordic may only be interested in producing salmon in saltwater, saltwater can be used to grow out eelgrass or saltwater vegetables for human consumption.

In the United States and Europe, there is work underway by commercial Land Based RAS operators like AquaMaof, as well as Universities to achieve zero effluent discharge through the use of tank repositories of microalgaes. These microalgaes produce materials that are used for either producing fish food, bio-fuels or cosmetic ingredients.

So whether by utilizing a licensed technology, or a hybrid of several methodologies, Nordic Aquafarms is able to achieve zero liquid effluent to the public waterways and greatly reduce their freshwater requirements.

Nor did the proponent offer an analysis of an aquifer water only based design using it's existing technology.

Nor did the proponent offer to use only local salmon genetics that have tested virus free and develop a less risky broodstock and eggs source as per the Williamsburg Treaty.

These are viable solutions and mitigation plans to the many risks associated with the current flow through design and its myriad of issues shown by the history of other projects in other jurisdictions.

Summary :

UVc only works if viruses are not shielded by particles in the water. This is affected by the size and the number of particles in the water called turbidity. The proponent assumes turbidity is constant, which it is not, as will become apparent after the first real storms. The proponent assumes that diverting from untreated reservoir water to sea water as an intake source will circumvent turbidity issues. Often, when storm make water turbid it affects both city water turbidity and water in adjacent bays that these turbid streams and rivers flow into. The proponents application shows no data to support the assumptions that diverting to seawater and using city water will nullify turbidity issues due to storms.

Discrepancies Between Intake Water Treatment and RAS/Processing Plant Effluent :

1. Drum filters are 10 micron on intake and 0.4 on effluent. If 0.4 is required to allow UVc penetration of the effluent it should also be required for the intake water.

2. Municipal water is simply carbon filtered without any micron filtration or UVc treatment, this assumes that town water treatment systems are designed to remove and are monitored for fish pathogens. This is a serious design error.

3. 10 micron drum backwash water is dumped in the sewer from the concentrated pathogen laden sludge generated by the intake water (illegal by Maine law). This is collected and disposed of in the effluent treatment.

We Are Still Finding and Learning about Salmon Viruses :

Finfish virology is a new and expanding science. New viruses are constantly being discovered and techniques for monitoring are still being developed for many viruses. Similarly new hosts are being found and many have unknown modes of transmission. One can't control what one knows so little about.

All RAS Systems Have Disease Issues, but Especially Those Using Surface Water :

Most regions have banned surface water use due to the unavailability of effective intake water filtration systems. A recent example is NS Canada after horrendous antibiotic use and disease outbreaks. No RAS system in Atlantic Canada has been recently built or licensed that uses surface water.

Import of Non-Native Fish :

The Williamsburg Treaty states that only local salmon should be used. The USA/Maine signed this treaty. Importing non-native fish often results in importing non-native viruses and other pathogens. Not all viruses are screened for in egg or smolt importation. Once imported and released it can never be taken back.

Lack of Fish Disease Control in the Food Supply System :

USDA, etc are only concerned with human pathogens. Even ISAv, IPNv, PRv, SAv, etc infected fish are allowed to be sold and washed down a sink. As long as the pathogen does not cause extreme mortality of near market ready fish, it is allowable. Effluent from such fish can be washed down any drain and allowed to be amplified in any RAS system. We have no science on the effect of such a disregard for wild fish exposed to fish produced artificially.

Lack of Antimicrobial Testing :

The USA does not adequately test RAS produced fish for antimicrobial resistance at the tank stage of production, nor the effluent.

Lack of Effluent Screening :

No government or independent monitoring of the effluent will be conducted for virus etc shedding.

Other Underlying Issues :

Lack of a pilot project. Issues in pathogen neutralization commonly seen in other semi-closed hatchery and RAS grow-outs such a biofilm issues eg Langsand Laks collapse due to disease, etc could be examined, and seasonal turbidity could be assessed.

What is “good enough” in terms of fish health regarding the stages of intake/import, husbandry, product and by-product is financially and human health driven with little regard to the Precautionary Principle or wild fish and wildlife health, which ultimately we are all dependent on. If a sick fish in a tank can gain weight, chances are it can be sold and washed down a drain.

What is “good enough” in terms of monitoring production. Unknown causes of mortality is the norm in RAS systems. In fact, 22% is a commonly factored-in mortality for fish from egg to just to smolt stage. Often, a further 20+ percent die as post smolts, eg Kuterra in BC Canada, Langsand Laks in Denmark, etc. The proponent has not included these mortality numbers in its effluent discussions.

What is “good enough” in terms of intake and effluent filtration. Again, acceptable levels of mortality is what is important, not fish health or exposure of pathogens via fish sold to the public nor effluent contamination.

When asked by the public about pathogens in the effluent water the company responded with (emphasis added by me):

“ I. Treatment and Containment of viruses and disease.

1. Specifically, how will disease, viruses and sea lice will be managed within the facility and prevented from being transmitted to Penobscot Bay where they could impact wild populations?

One of the major benefits of RAS is the ability to control the culture environment and prevent disease. All egg batches will be sourced from a **reputable breeder** with **a staff veterinarian supervising a routine screening procedure for salmon diseases**. Upon receipt, eggs will be further screened and quarantined in collaboration with **independent fish health experts**. The most likely source of disease risk would be the sea water used. **All water** entering the facility will be treated with **ultra violet (UV) light** (see Attachment F) using technology that is **proven to neutralize parasites, bacteria and viruses**. The internal RAS system will **continuously treat the recirculating water; preventing the growth of any pathogens within the RAS system**. Finally, all water leaving the facility will be treated

with **membrane filters and UV as well**. We will also work with a **licensed veterinarian**, who is experienced in aquaculture, to assist us in adapting our established biosecurity measures to **US requirements and conditions**."

The key words here are "reputable breeders". No names are given and most breeders and egg suppliers regularly are caught shipping and amplifying viruses in their fish. This will be discussed below.

The next key phrase is "routine screening procedures" using a "staff vet". This certainly sounds like a made up screening list and protocol that may not include all potential pathogens and may not use the latest techniques and methods. No biosecurity protocols or testing methods are given. This is a huge issue.

Also of note is that "independent fish health experts" means paid staff again, but under contract instead of direct employment.

The company then proceeds to suggest that the UV light technology they will use will neutralize pathogens but does not give any evidence nor the degree of neutralization.
On page 223 of the MEPDES permits see here: https://www.maine.gov/dep/ftp/projects/nordic/applications/MEPDES%20Permit%20Application_Final_Oct%2019,%202018.pdf
the company states:
" The internal RAS system will continuously treat the recirculating water; preventing the growth of any pathogens within the RAS system." This ignores fish to fish transmission within the tank long before the water is hit by any filtration or UVc. Once a pathogen is in the tank the UVc and filters are almost ineffective in preventing the spread of any pathogens. The proponent talks as if their is a filtration system between each fish. This of course is ridiculous.

To actually dismember the viral RNA takes much higher doses of UVc than that required for a mere 3-log reduction, Oye and Rimstad 2001. Skall and Olesen 2011 reviewed all methods of viral inactivity for fish processing plants and were unable to filter the water and disinfect it with UVc. Moreover, they showed just how dramatic turbidity can affect the UVc.

The proposed pathogen reduction system also ignores airborne transmission such as seen with water molds that can devastate RAS facilities (Sakaguchi et al 2019). This was elucidated only months ago despite being a multimillion dollar issue for decades. This speaks to just how little we know about rearing healthy fish in captivity.

Often UVc is only used to determine the 99% - 99.9% reduction in pathogens in the literature, (Torgersen and Hastein 1995). This is referred to a 2-log and 3-log reductions in the percentage of pathogens. Not absolute numbers but percentages, which of course, are relative to how many pathogens are in a system. 100% reduction (ie sterilization) is never seen in an operating RAS system. UVc highly resistant viruses like IPNv (Liltved et al 2006) often cause limited to no mortalities in hatchery RAS salmon but due to this limited financial impact can result in ignoring it in broodstock and in an RAS setting. IPNv resistance to UVc treatment (Liltved et al 2006), is nearing the limit of the proponent's treatment dose under ideal conditions ie 246mJ sec cm2 in seawater vs 300 mJ sec cm2 offered by the proponent. To reduce IPNv by a 6-log reduction (99.9999%) requires >800 mJ sec cm2 (Skall and

Olesen 2011). Viral particles are counted in the billions per liter in some cases in tanked RAS fish. 800 mJ sec/cm2 is two and a half times above the proponents treatment threshold . This is also likely why IPNv is so prevalent in hatcheries at low levels.

Small changes in turbidity can seriously reduce the effectiveness of the UVc treatment.
Moreover, once in the system, pathogens not requiring an intermediary host can be amplified by fish to fish transmission exclusive of the UV treatment restricted to a UV pipe the fish never enter.

The company mentions continuous treatments during the recirculation process but does not mention how this will work nor how effective it is against viruses and other potential pathogens but states it will use membranes and UV. Percent effectiveness of each treatment is not given.

The company then suggest that it will hire a vet and make sure it follows the laws regarding pathogens in effluent. There are no effluent virus laws, nor any monitoring. No company has even been convicted of dumping fish viruses in the ocean via effluent in USA of Canadian history, that I am aware of. This is telling. This is despite that every farmed salmon in the USA is washed down a sink and has a very very high probability of having a heavy viral load. Eg a alck of PRv testing in Washington in Atlantic salmon produced by Cooke (recently banned due to pressure from NGOs).

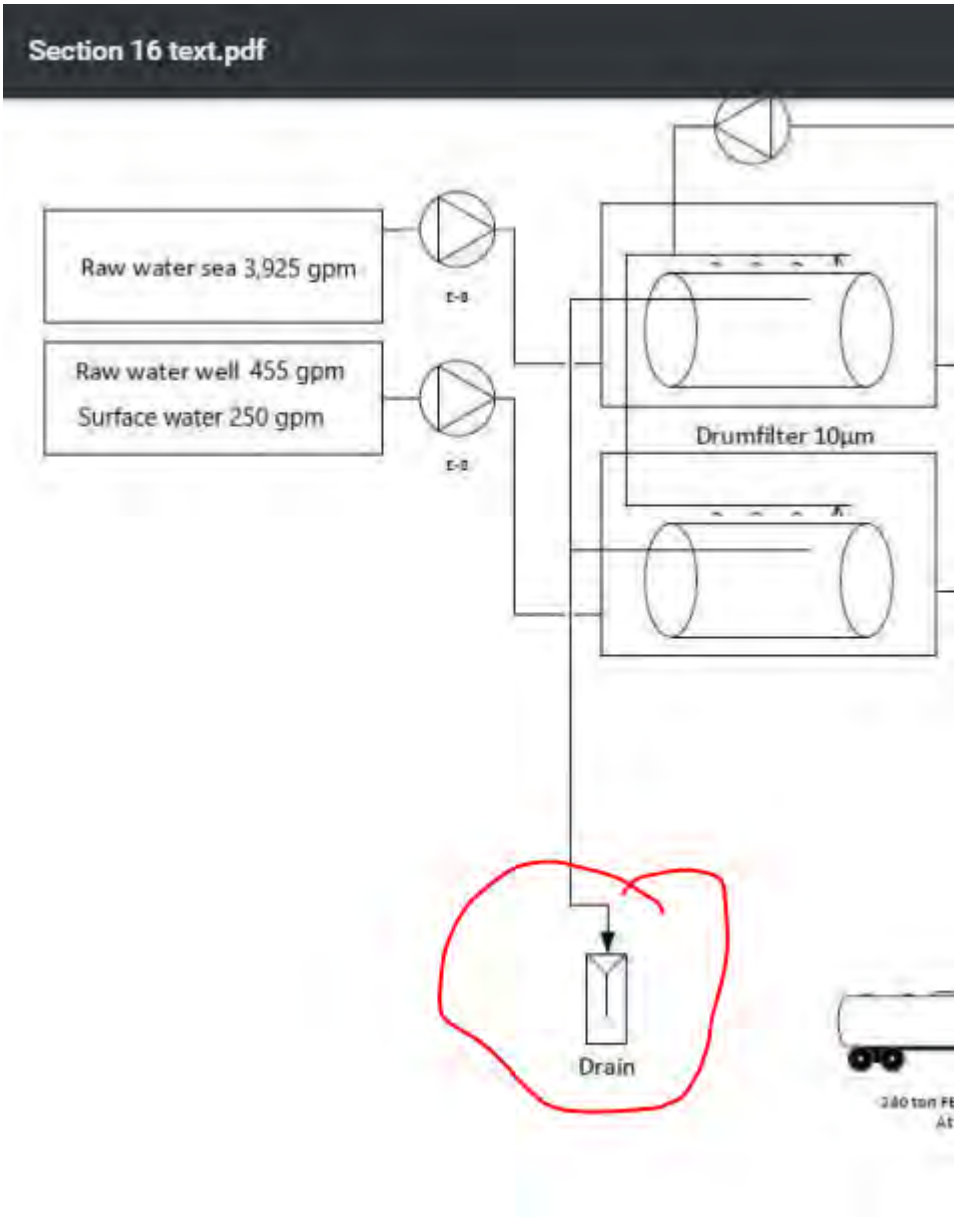
Any discharge of viruses would be covered, we hope, under Chapter 800 of the Maine State Environmental Protection which covers Hazardous Matter. This could require a complete cessation of the effluent but the system design does not sensibly allow for this. Nor is a “biblical scale” 33,000mt depopulation plan submitted that would handle tens of millions of salmon.

Water Intake Issues :
Right from the outset the proponent demonstrates that impacts to wild fish health are not a priority.

The pathogen concentrate ie the backwash sludge from the 10 micron drum filters on the intake pipes is simply dumped into a drain. This will invariable concentrate pathogens to levels that were not previously at densities that typically allow pathogen transmission in the intake water. This sewer pipe then acts as a major disease vector for ALL pathogens in the entire Little River and Bay water ecosystems. The plant become a disease concentrator.
This is not what the proponent told the public. “Just a final step in terms of the treatment process. So there are solids coming out of various parts of this treatment process. All these solids go through a special dewatering process and what you end up with is a sludge very high and rich in nutrients. And this sludge is in Maine's case going to biogas production. Other cases we see, for example, Norway, it's used for fertilizer, and other types of projects, but in Maine we are sending it to biogas production. And that basically means none of this is going into the ocean.
It's being recycled. “ pg 238 DEPLW1999-19

The design of the intake of surface water and aquifer water is outlined on pages 2 and 3, seen here:https://www.maine.gov/dep/ftp/projects/nordic/applications/SLODA/Section%2016%20-%20Water%20Supply/Section%2016%20text.pdf

A 10 micron filter is used to remove large solids. This is followed by a brief blast of ozone to bleach some of the solids. UVc is then used at a suggested 250-300mJ/cm2 at 85% transmission rate to kill viruses.

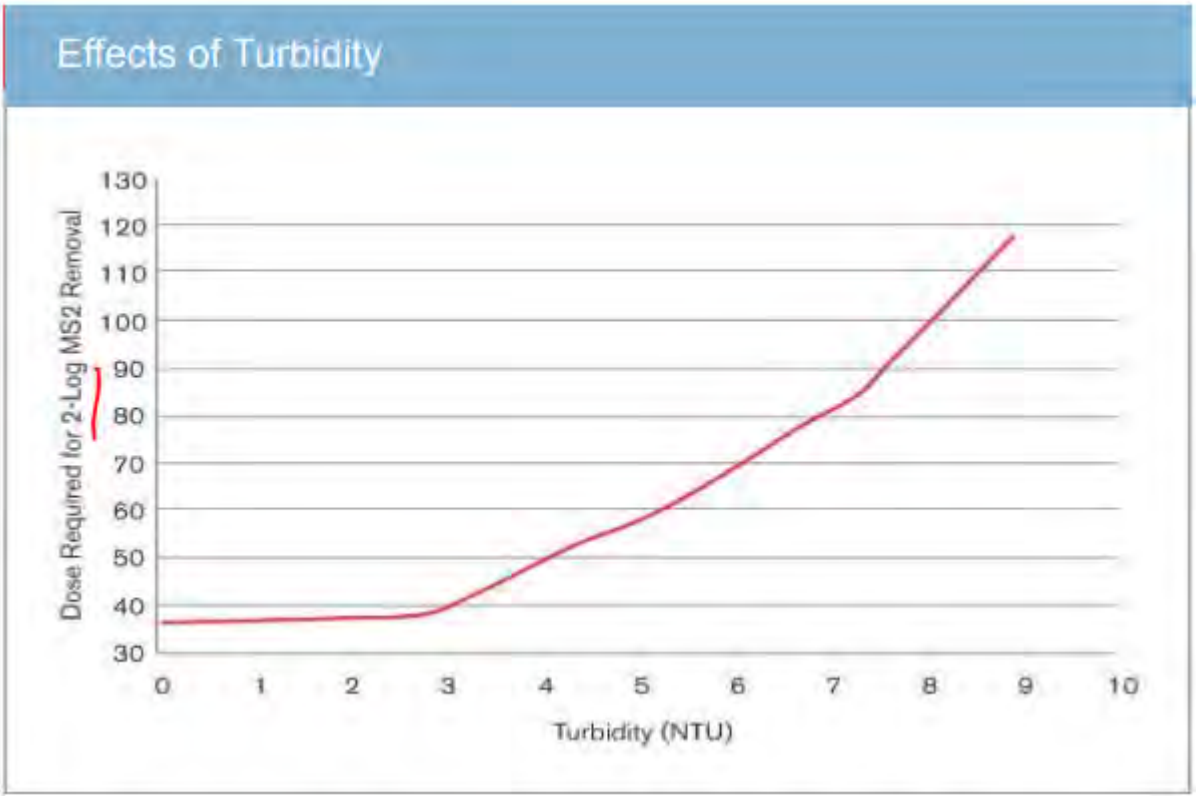


This 85% transmission is totally dependent, as admitted to by the proponent, upon a constant water turbidity as seen under laboratory conditions. This is what the manufacturer and scientists would have used to evaluate the amount of UVc lamps needed. These conditions of course are never the case in a living river with turbidity fluctuations of up to two or more orders of magnitude. The proponent suggests that this will be compensated for by using more seawater. Ironically, typically during times of freshwater extremes in turbidity this added turbidity simply end up travelling for miles throughout an adjacent bay.

The only solution to reduce the turbidity would be to revert to only using aquifer water. Even the town water will increase dramatically in turbidity at times. Note the **log 2 reduction** (only 99%, not 99.9%) in the graph supplied by a competing water pasteurization company. https://www.aquafineuv.com/cms-portals/aqua_com/cms/static/documents/Presentation-Pasteurized-Equivalent-Water-by-Ultraviolet.pdf

The town water is simply carbon filtered by the proponent. This is despite that the town water is not designed nor mandated to be “fish” nor “RAS safe” (Head/Superintendent of Belfast Water District, Keith Pooler per comm. Dec 9 2019) Pathogens that affect fish health are ignored in the town water treatment design (Keith Poolers per comm Dec 9 2019). Moreover, Mr Pooler suggested that neither UVc nor any micro filtration is part of the town water supply treatment. Furthermore, that the town does not guarantee the water quality provided to the proponent, nor, to his knowledge has the town every conducted any long term testing or pilot project to challenge fish with town water. I suggest that

this



water supply may, at times of extreme drought and surface water ingress, contain fish pathogens. Mr Pooler stated that at times of severe drought that the aquifer water may increase in turbidity and that this is due to surface water infiltration into the aquifer at high enough rates to cause an increase in turbidity, though this is rare. I would suggest that if turbidity is increasing due to surface water entering the town water system then surface water viruses are also likely entering the system and that this has never been monitored nor tested.

Mr Pooler was also able to confirm that a maximum limit of 262 million gallon of water per year offered to the proponent was the safe limit of the existing pipe system owned by the town. Moreover, that while the aquifer could offer perhaps up to 600 million gallons, that the pipe system would need serious upgrades. The current design and desired production capacity of the proponent is an order of magnitude above what the town can currently supply. >2.81 billion gallons per year vs 262 million gallons per year. About 1/10th. "Right sizing" the project to, as the proponent suggested, "reducing the risk of pathogens entering the system by using the best quality water available in term of pathogen sources" would require the down scaling of the project to 1/10 of the 33,000mt desired by the proponent. Or, the town water supply system getting an upgrade likely via funds/taxes/etc levied on or supplied by the proponent.

Of course only using aquifer water, ie "right sizing the project", is a suggestion the Authorities need to seriously discuss as an option with the proponent.

When asked by the public:
"I. Viruses/disease:

1. "2018, CBC news reported "Virus at 2 Nova Scotia land-based fish facilities results in 600,000

> salmon being killed ... Aquaculture Minister Keith Colwell said Thursday the two facilities are located close to each other but wouldn't name them." If Nordic has a disease outbreak, will it be required by law to disclose the location to the public?

Nordic will follow all reporting requirements in the U.S. and Maine. We cannot speak to the biosecurity measures of these two Canadian facilities. Nordic Aquafarms will install significant upgraded biosecurity measures compared to most of the industry in addition to implementing our best practices for land-based operations, to prevent pathogenic material from entering or leaving the facility. We are not a net pen operation putting fish into the ocean."

What "significant upgraded biosecurity measures" the proponent suggests are unclear at best. Surely not UV light and drum/membrane filters? This is ancient/well established technology. Further statements in the answer to this critical question are vague and uninformative such as "best practices".

The company then makes several serious errors in logic and when asked about viruses when stating:

"*If you have a disease or virus outbreak, will the tanks continue to circulate the disease into Penobscot Bay?*

Pathogenic materials will be unable to enter or leave the facility. ***The primary source of pathogens for RAS facilities is the water source they use.*** *We will use* ***proven disinfection technology*** *at our intake to prevent pathogenic material from entering the facility. The tanks circulate on an internal water* ***treatment loop that has UV disinfection*** *integrated into the RAS for continuous disinfection of system water. Grow-out and processing tanks drain to a waste water treatment system that has micro-filtration to remove particles* ***as small as 0.4 microns*** *(a human hair is 50 microns). This is small enough to remove bacteria. For comparison, rod shaped Escherichia coli bacteria are 1 micron by 2 microns in size. After micro-filtration water is treated with a 300 mJ/cm3 dose of UV light for final disinfection prior to discharge.*"

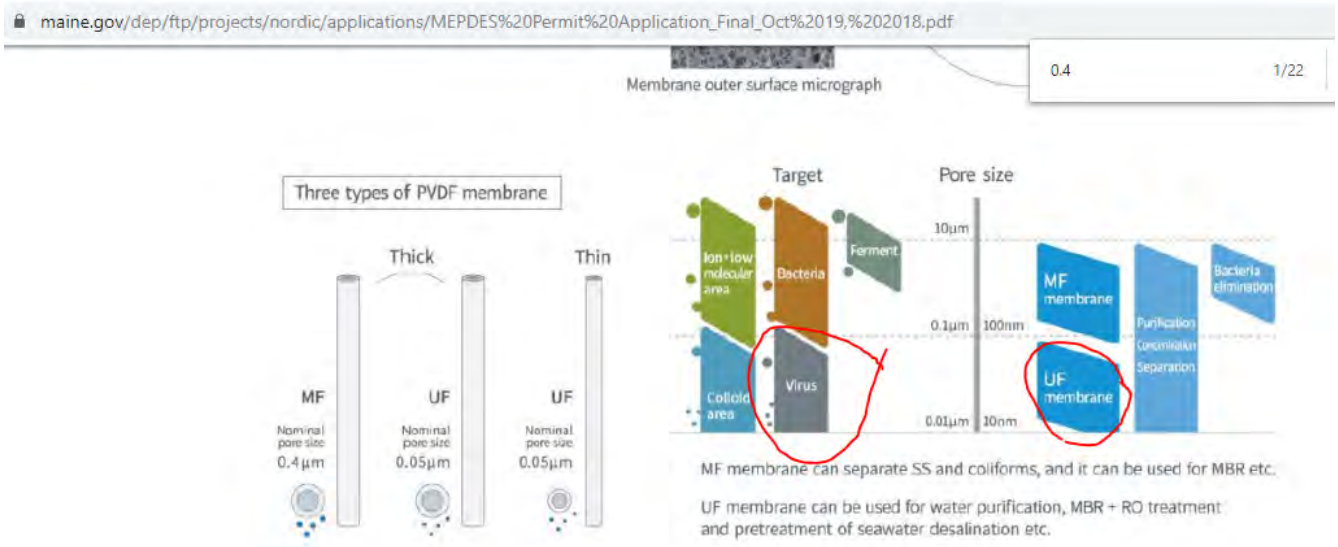
They state that " **The primary source of pathogens for RAS facilities is the water source they use."** This is very true, yet the company ignores a production model that reduces this by simply only using aquifer provided naturally filtered water that is virtually free of viruses. This is truly telling about the managers risk vs profit assessments. Many viruses only cause minor profit losses via protected tanked fish that are sick with a non-OIE reportable disease. This can not be said for wild fish exposed to such viruses from the effluent in a wild/predator environment.

The company then goes on to suggest that drum and membrane filtration down to 0.4 microns will be used and then uses bacteria as a measuring stick but fails to mention that viruses are measured in nanometers (1000 times smaller than a micron). Then, it suggests that a millisecond blast of UV light is effective at eliminating viruses, without discussing those shielded inside particles. This is far from the truth as evidenced by the dozens of hatcheries using UVc globally that suffer from pathogen outbreaks and extreme antibiotic use. It does not state the percent filtration rate at 0.4 micron ie how many particles larger than 0.4 microns will get through per minute, nor suggest evidence of this testing. No filter is perfect.

The company also suggests that the TSS will be 1.5% of input water values, but in reality, this is not constant as the filter becomes fouled and the water turbidity changes. The 1.5% is what the manufacturer observed under constant laboratory ie "ideal" conditions. The first storm that hits Maine will show how much this can change in the real world.
See page 73 of the PDF at https://www.maine.gov/dep/ftp/projects/nordic/applications/MEPDES%20Permit%20Application_Final_Oct%2019,%202018.pdf

While the company admits that a filter from the same supply company is available that is somewhat effective against viruses (see screenshot below), the proponent's design does not use it but instead uses 0.4 microns which will not filter any viruses and instead opts for the cheaper than ozone or membrane filtration - UVc treatment. The problem with virus control in water is that as the system design become more effective it also becomes more expensive regarding filters and or chemical use. It seems that in various discussions with various folks (Dr Dixon, Mr Lannen, etc) people are surmising that a 0.1 micron is the filter they should use to treat seawater coming in (Dixon written submission).

Apparently, when Mike Lannen questioned them on a "circle" that was in their schematic documentation where a filter would be and asked "what is this" the Nordic representative refused to answer. I believe the proponent must supply this info to DEP if asked.



The company then lists mildly or totally unrelated peer reviewed papers on UVc and various parasites that the 0.4 micron filter should remove - among which not one is an original piece of work examining their system on even a bench top laboratory scale. No test of efficacy of their system is offer in any way. Often, expensive blood and bacteria filters fail, become plugged, etc and are bypassed. This is often the case and has been admitted to by facilities in NL via government emails to me.

In fact, 22% mortalities is a common benchmark for ultra modern hatcheries eg Grieg in NL Canada; Cooke Aquaculture hatchery in St Alban's NL, or the Marine Harvest hatchery in Stephenville, NL that are designed to or use aquifer only water, micron scale filtration and UVc treatment - and all suffer from regular pathogen and virus issues. Both of the main hatchery in NL have serious and continual pathogen issues including viruses (eg ISAv), fungi, and bacteria. This is verified by the mortality rate and the following ATTIPA response FLR/75/2018 by the NL government agency responsible an excerpt of which is seen here:

The “no regulatory response required” to ISA HRP0 in the hatchery is telling and normal for NE North American hatcheries. HPR0 is thought to quickly become virulent (see Are Nylund etc quotes below regarding this issue in hatcheries). Moreover, how did a virus get into a land based aquifer only water hatchery? Ans. It was imported. ISA HPR0 and may other viruses are rampant in NE North America hatcheries. See ICES reports on diseases and parasites for examples from 2015-2018.

Site	Company	PCR results	Sequencing	Status	Year
Olive Cove	COS	HPR0	HPR0	No required regulatory action	2017
Grip Cove	COS	HPRΔ	RPC# 23	Quarantine Order - site depopulated	2017
The Matchems	COS	HPRΔ	RPC# 23	Quarantine Order - site depopulated	2018
Spy Glass Cove	MH	HPRΔ	RPC# 11	Quarantine Order - site depopulated	2018
Stephenville Hatchery	MH	HPR0	HPR0	No required regulatory action	2018
Robin Hood Cove	COS	HPRΔ	RPC# 23 and 18	Quarantine Order - site depopulated	2018
North West Cove	COS	HPRΔ	RPC# 25 and 26	Quarantine Order - currently depopulating	2018
Tilt Point	MH	HPRΔ	RPC# 11	Quarantine Order - site depopulated	2018
McGrath's Cove South	MH	HPR0	HPR0	No required regulatory action	2018

Legend

COS	Cold Ocean Salmon
MH	Marine Harvest (Northern Harvest Sea Farms)
HPR0	Non-pathogenic - not known to cause disease
HPRΔ	Pathogenic ISAv

Ultraaqua is a USA leading water disinfection supply company with hatchery clients in 50 states. It admits on its website that only a percent of pathogens are neutralized by any system. That this percentage is proportional to the amount of pathogens in a system, and finally, that turbidity, which can dramatically plug or otherwise negative impact the efficacy of a UV and filter disinfection system, is variable and seasonal in nature.

Disease causing agents can short circuit the system by simply passing from animal to animals without passing through the filtration system. The result is a build up of pathogen production in the tanks. At a theoretical limit the feedback loop reaches a pathogen production value that results in enough pathogens to simply pass through the system and infect significant number of animals through the disinfection treatments system. Spotti and Adams 1981 reviewed this mathematically.

UVc would be used by the proponent at an ideal rate suggested at 250-300mJ/cm2 at 85% transmission rate to kill viruses and other pathogens. This is very close to the limit for many known and common hatchery viruses as seen in the table below. It is also not nearly enough treatment for highly resistant pathogens like whirling disease which require a minimum of 1300 mJ/cm2 (Hedrick et al 2000). Four times that of the proponent's design. Most town water treatment systems will also not neutralize cryptosporidium. This pathogen is also at about the water intake filter limit in spore size ie 10 microns.

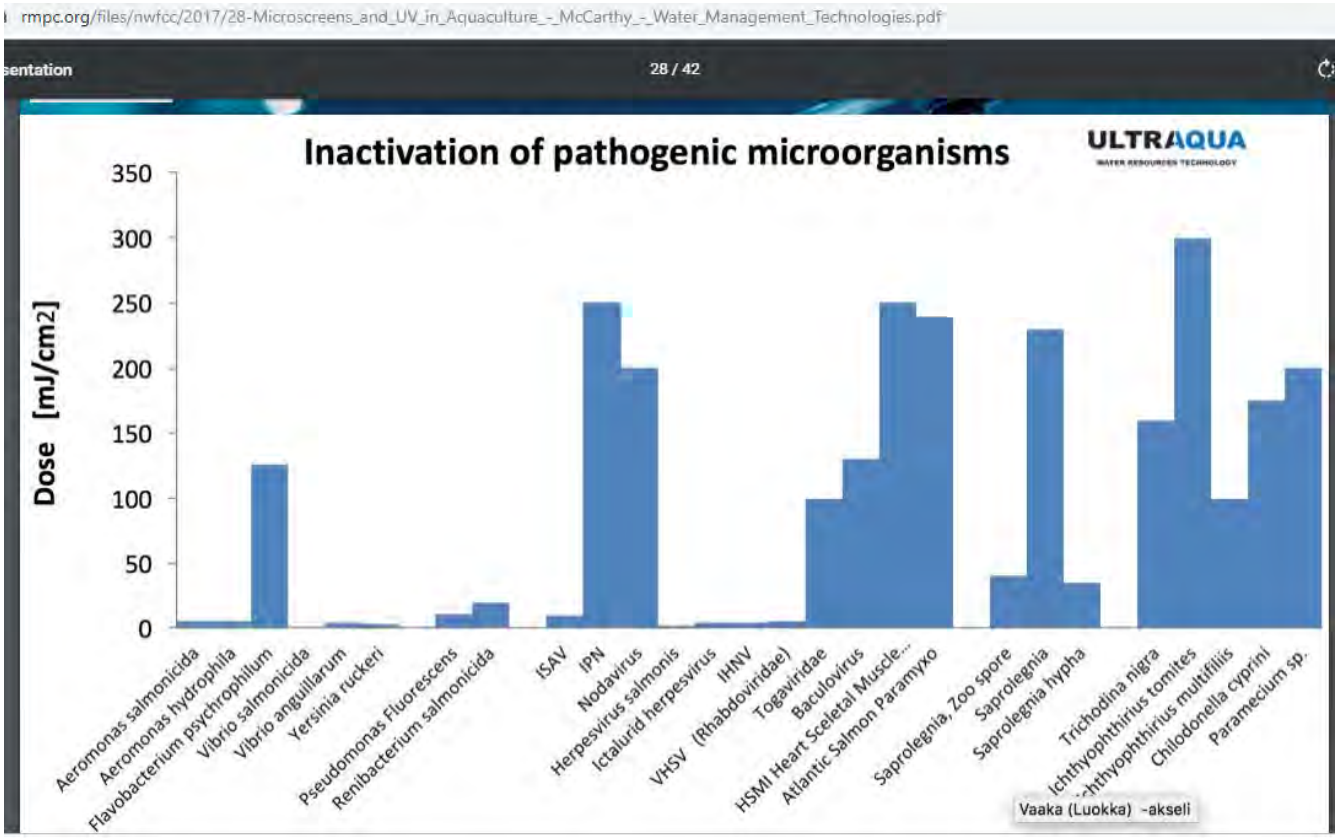
Similarly, even higher doses (400mJ/cm2) have been shown to be insufficient to sterilize intake water against water molds (*Saprolegnia spp*) that cause serious losses in hatcheries (Heikkenin et al 2013).

Disease thought to only be transmitted in saltwater are also being discovered in freshwater. Systems and their resulting monitoring are generally not designed to consider anything beyond what is currently known to exist in freshwater hatcheries. SAv, a leading cause of marine salmon losses in the aquaculture industry, was only recently recognized to be an issue in freshwater hatcheries (Soares et al 2019). Note that the hatchery was breeding this disease for a unknown time and was unable to diagnose the pathogen.

The suggested UVc needed for pasteurization varies highly between companies and often is not

supported in the scientific literature. This is a serious issue mentioned in many peer reviewed publications eg Liltved 2006, Skall and Olesen 2011.

Eg of the limitations of current testing and models for a leading UVc based system, only a small percentage of pathogens are listed and many are near the upper limit of the proponents UVc dosage:



Recently, after spending circa $250 million dollars trying to prevent ISAv from entering the food chain the CFIA (Canadian Food Inspection Agency) gave up on containing ISAv. It was found repeatedly in hatcheries throughout Atlantic Canada, and the open net pen industry had gobbled up more than 98% of all taxpayer funded “crop insurance” provided to farmers for any and all types of food produced in Canada – two years in a row. This was decidedly unpopular and unsustainable. Instead of shutting down the open net pen production of salmonids in Canada, the CFIA then removed finfish aquaculture from this taxpayer funded “crop insurance” program and suddenly produced evidence that the fish were safe for human consumption. The finfish aquaculture industry was no longer a crop but a fishery. Ironically, or sadly, the Federal agency responsible for protecting wild fish, the DFO, had washed their hands of this responsibility and provided disease oversight solely to the CFIA. Today, and unbeknownst to the public, there is a warning on a buried federal website stating that ISAv infected salmon should not be washed down a drain. https://www.inspection.gc.ca/animals/aquatic-animals/diseases/reportable-diseases/isa/fact-sheet/eng/1327198930863/1327199219511 It may also be of interest to know that ISAv was misdiagnosed in Canada for more than 2 years before it was properly diagnosed for the first time in 1998. Massive industry crippling outbreaks began in 1996. A similar scenario exists for most

salmon viruses in aquaculture production. In fact, despite being present for nearly 2 decades, three new crippling viruses were announced to science mere weeks ago. Weeks ago, another thought to be benign was shown to be extremely pathogenic (PRv via HKS with ISA) and a third was found to be able to reproduce in freshwater (SAv). These will be discussed below.

In fact, when I questioned the USDA and Maine government about this issue via Dr Thomas McKenna and Dr David Russell (vets for their respective agencies) in 2018 they referred me to Jeff Nichols of Media relations for the Maine State Marine Resources. I assume the actual questions were answered by Dr Russell. So Jeff suggested that Maine is not concerned about ISAv infected fish being imported from Canada and that Maine has decided this is not an issue at all. See the email response in red in the screenshot below. This, despite two decades of not allowing this to happen. Most notable, this sudden and 180 degree shift in policy occurred *immediately after* to the industry being cut off (2013/2014) the disease compensation for the 5 OIE viruses that includes ISAv. Once cut off the free taxpayer provided (in Maine and Canada alike, using the exact same protocols) "cull insurance" the ISAv infected salmon were suddenly allowed in the USA. The industry was too big to fail at this point it would seem.

Does your government have any studies to show that the risk of consumers spreading the virus via typical consumer practices is extremely low? Or, studies/monitoring that show that current policies and mitigation measures and public awareness is controlling the potential for spread effectively? Seems that having an intensive costly fish health surveillance program and a potentially costly compensation package available due to cull orders is extreme given the protocol is to allow shipping it to 100s of 1000s of drains all over America, including many attached to rivers and estuaries (without any treatment) inhabitated the NOAA posterchild for endangered species - wild Atlantic Salmon. In Maine, most food handling drainage enters septic tanks or publicly owned sewer treatment plants that are not conducive to ISA survival and transmission to a susceptible host at an infective dose. The amount of virus shed from household washing of small amounts of infected product to a private septic system or to a municipal waste water treatment facility, when factoring in dilution, and loss associated with biological and chemical activity results in a negligible risk of transmission to a suitable host at a dose likely to be infective.

https://www.ncbi.nlm.nih.gov/pubmed/24689956

Research has also shown that ISAV does not replicate at temperatures of 25C and above. It is inactivated quickly at such warm temperatures. As such, It is not viewed as a threat for humans.

https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scah_out44_en.pdf

I know there is strong interprovincial agreements on how to best manage ISAv in Canada (one crew decides kit all for everyone), and basically everyone in Canada is implementing the same strategy, I am curious to know if Cooke, Cooke's paid consultants, and the USA government ISAv managers are agreeing to work hand in hand with Canadian managers such that both Maine USA and Atlantic Canada are implementing the same protocols and securities? We work cooperatively with the Canadian government to ensure testing methods, response, biosecurity, and fallowing are coordinated between jurisdictions.

The issue with this response is obvious. No studies to prove the statement made about washing it down a drain have ever been completed in Maine or anywhere else. This is telling.

Moreover, the issues with the industry (Cermaq) funded study are many. Industry had provided this study that "proved" that UV light kills ISAv quickly and easily. The issue with the study is that the virus was not IN the fish or even its mucus, it was loose and added to the effluent water. This provided limited protection to the virus from the light. There were also serious issues with media used that destroyed entire sample treatments, tiny sample sizes of 2 fish, and more issues with the study. These flaws in the design of the unnatural study using UVc lamps to simulate sunlight is ignored by governments. Real world tests of fish infected with any of the known viruses that do not require an intermediate vector invariable results in the control fish becoming infected, even under natural UV light conditions in a tank. This UV light experiment cited by the Maine government regarding this issue is Vike et al 2014 and found here:

https://afspubs.onlinelibrary.wiley.com/doi/abs/10.1080/08997659.2013.864720

Norway is now so worried about the recent return of this ISAv to its open net pens that it has reinstated a 10 Km radius around an infected sites whereby all adjacent sites must be culled. This will cost 10s of millions of USD every time it is done. So far, 4 outbreaks have been recently reported and this "stamping out method" is now in force. Salmon are a pelagic species typically found within 2 fathoms of the surface. A salmon gill net is almost never more than 2 fathoms deep. If this virus was so easily killed by UV light, how could it ever be passed from one fish to the next so close to the surface in gin clear open ocean water? Moreover, the last Author of the study above now recommends stamping out all ISAv HPR0 in all broodstock fish. He also suggests, along with the top vets in Norway that this is a serious issue in hatcheries supplying eggs to facilities such as the one proposed by Nordic Aquafarms and has been an issues for decades. This will be discussed below.

The company then suggests that the filtering technology is proven. Where is this proof? Every hatchery in the world to date has contamination issues. Especially those using surface water, drum filters and UV light for pathogen control. On page 207 of the MEPDES application the proponent admits that there are no technical evaluations nor any pilot projects or test data that are available regarding the effluent. Belfast will be the first guinea pig and on an unprecedented scale of 33,000mt of production and 7.7M gallons of effluent a day spilling into the last stronghold of a species supposedly enjoying the protection of the Federal Endangered Species Act in Maine. https://www.fisheries.noaa.gov/species/atlantic-salmon

VI. Engineering Report on Wastewater Treatment

A. If there is any technical evaluation concerning your wastewater treatment, including engineering reports or pilot plant studies, check the appropriate box below.

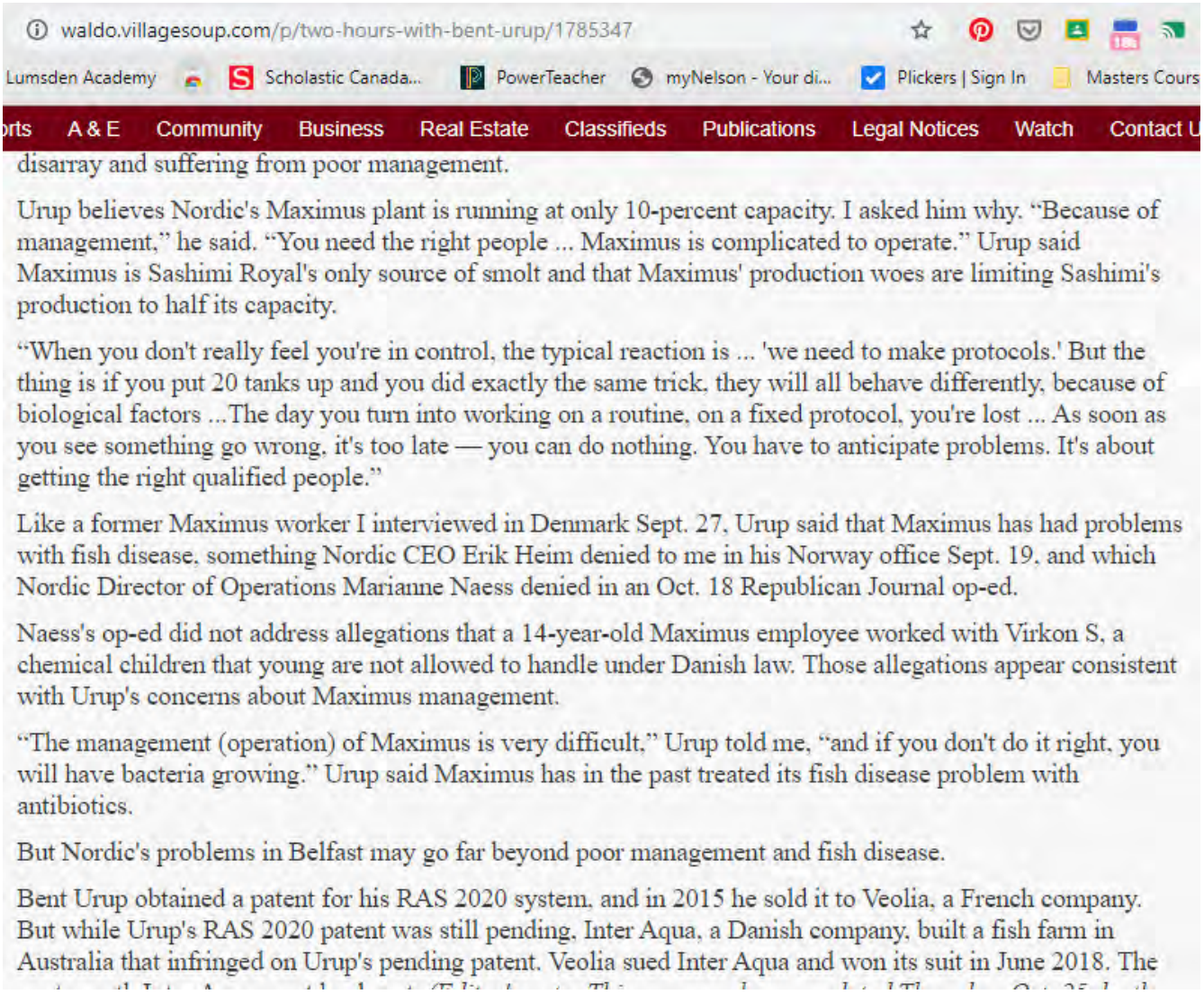
☐ Report Available ☑ No Report

B. Provide the name and location of any existing plant(s) which, to the best of your knowledge resembles this production facility with respect to production processes, wastewater constituents, or wastewater treatments.

Name	Location
Sashimi Royal	Nordre Strandvej 66, 7730 Hanstholm, Denmark See Attachment 3 for Sashimi Royal water quality results.

EPA Form 3510-2D (Rev. 8-90) Page 4 of 5 CONTINUE ON NEXT PAGE

Regarding the proponent using Sashimi Royal as a track record, it's first cohort was just harvest in summer 2018, which hardly gives this very small facility much of a history. I am also lead to believe that extensive antibiotics were also used to rear the fish and mortality rates were extreme. This does not include a likely lawsuit and discouraging remarks from RAS 2020's designer. https://waldo.villagesoup.com/p/two-hours-with-bent-urup/1785347



waldo.villagesoup.com/p/two-hours-with-bent-urup/1785347

Lumsden Academy | Scholastic Canada... | PowerTeacher | myNelson - Your di... | Plickers | Sign In | Masters Cours

orts | A & E | Community | Business | Real Estate | Classifieds | Publications | Legal Notices | Watch | Contact U

disarray and suffering from poor management.

Urup believes Nordic's Maximus plant is running at only 10-percent capacity. I asked him why. "Because of management," he said. "You need the right people ... Maximus is complicated to operate." Urup said Maximus is Sashimi Royal's only source of smolt and that Maximus' production woes are limiting Sashimi's production to half its capacity.

"When you don't really feel you're in control, the typical reaction is ... 'we need to make protocols.' But the thing is if you put 20 tanks up and you did exactly the same trick, they will all behave differently, because of biological factors ...The day you turn into working on a routine, on a fixed protocol, you're lost ... As soon as you see something go wrong, it's too late — you can do nothing. You have to anticipate problems. It's about getting the right qualified people."

Like a former Maximus worker I interviewed in Denmark Sept. 27, Urup said that Maximus has had problems with fish disease, something Nordic CEO Erik Heim denied to me in his Norway office Sept. 19, and which Nordic Director of Operations Marianne Naess denied in an Oct. 18 Republican Journal op-ed.

Naess's op-ed did not address allegations that a 14-year-old Maximus employee worked with Virkon S, a chemical children that young are not allowed to handle under Danish law. Those allegations appear consistent with Urup's concerns about Maximus management.

"The management (operation) of Maximus is very difficult," Urup told me, "and if you don't do it right, you will have bacteria growing." Urup said Maximus has in the past treated its fish disease problem with antibiotics.

But Nordic's problems in Belfast may go far beyond poor management and fish disease.

Bent Urup obtained a patent for his RAS 2020 system, and in 2015 he sold it to Veolia, a French company. But while Urup's RAS 2020 patent was still pending, Inter Aqua, a Danish company, built a fish farm in Australia that infringed on Urup's pending patent. Veolia sued Inter Aqua and won its suit in June 2018. The

The responding affidavit by a company vet is extremely weak, cites extremely high mortality rates of 80-90% post hatching, and does not mention anything about proper genetic virus testing, but instead only far more subjective physical symptoms. Physical symptoms are not always present in viruses and some fish can act as carriers. Moreover, virus shedding is almost always greatest before physical symptoms occur, so all testing done based on symptoms is ad hoc in nature. The speed of immunological response can also not be related to virulence, eg McBeath et al 2014. The litmus test for viruses is not the physical appearance of the fish but rather PCR genetic analysis with appropriate primers. Sadly, there are not even universally accepted primers for most viruses, so avoidance of a positive detection can be done. No explanation of the high mortality rates are given. Fungus are also not mentioned. https://www.cityofbelfast.org/DocumentCenter/View/2297/20181018-veterinarian-affidavit-re-maximus?bidId=

It is also not salmon or even a fish native to the region that is being reared. Instead it is a tropical king fish. This is the nearest comparison the company could make to a new 33,000mt facility the likes of

Veterinary evaluation of Maximus A/S, 7755, Bedsted Thy and Sashimi Royal A/S, Nordre Strandvej, 7730 Hanstholm, Denmark

The facility Maximus A/S produces fish for on-growing at Sashimi Royal, Hanstholm, Denmark.

The survival rates at Maximus A/S are in the same range as other marine hatcheries, and mortality of weak and damaged larvae is not unusual. Survival is usually 10-20% of hatched eggs.

Maximus A/S has been checked by us, macro- and microscopely by DTU-VET (Danish official Laboratory for fish disease, also EU-reference Lab), and no virus was found.

Fish and larvae have been checked by us, macro- and microscopely, monthly since 14. December 2015, and no signs of disease, neither bacterial or parasitic infections has ever been detected.

At Sashimi Royal A/S, the fish has been continuously chekcked for diseases and parasites since the start of June 2017. Similar to Maximus no signs of diseases have been found that are attributable to bacteria, viruses or parasites.

Dr.Med.Vet Thomas Clausen,
Veterinary specialist in aquaculture
Ø.Høgildvej 12
7400 Herning
Denmark

which it has never operated nor tested. This is telling.

Page 210 of the PDF document for the MEPDES permit shows:

Gross / Net Discharge Figures - Belfast, Maine Facility

The table below summarizes our gross discharge of nutrients (before waste water treatment) and net discharge (after treatment).

Discharge Budget: 33 000 MT production of Atlantic Salmon, Nordic Aquafarms, Maine

	TSS	BOD	Total N	Total P	NH3	Unit
Smolt						
before treatment	226,748	198,405	68,248	17,118	2	kg/year
after treatment	2,267	1,984	10,237	171	2	kg/year
Phase 1 PB						
before treatment	3,235,594	2,831,144	778,939	96,535	12	kg/year
after treatment	32,356	28,311	116,841	965	12	kg/year
Phase 2 PB						
before treatment	3,235,594	2,831,144	778,939	96,535	12	kg/year
after treatment	32,356	28,311	116,841	965	12	kg/year
Processing Facility						
before treatment	57,143	62,857	10,400	859	1	kg/year
after treatment	571	629	1,560	9	1	kg/year
Total						
before treatment	6,755,078	5,923,551	1,636,527	211,048	27	kg/year
after treatment	**67,551**	**59,236**	**245,479**	**2,110**	**27**	**kg/year**
	185	**162**	**673**	**5.8**	**0.07**	**kg/day**
Concentration	6.33	5.55	23.0	0.20	0.003	mg/L
WWTP degree of removal	99%	99%	99%	85%	0%	

a 185 kilos a day suspended solids will be created. That is a lot of material to hide some viruses in from UV light treatment. A virus weights circa 0.000000000000001 kilos or there abouts as dry weight.

No land based RAS system, to my knowledge, has actually been successful in rearing salmon using surface water without serious, crippling, and virtually bankrupting in most cases, pathogen introductions. There are reasons microbiological labs autoclave anything they want free of viruses, bacteria, etc and even after that use hepa filtered air in positive pressure air supplied rooms, masks, gloves, etc and yet still have to deal with contamination issues regularly. I manage a “clean room” similar to a surgical room that develops new cultures of microorganism under these types of conditions and can assure the reviewers that the system designed by Nordic Aquafarms will have serious issues with pathogens that are 1000s of times larger than any salmonid virus. This is why they have listed so many antiparasitics and antimicrobial drugs in their project description. They are expecting to have to deal with these microorganisms. Many fish viruses are in the order of 30-100nm. A nanometer is 10^{-7} of a cm (less than a billionth of a 1/2 inch). 100s would fit on the width of a human hair.

There simply is no system that humans have designed that can filter more than 750 gallon per minute of freshwater, nor nearly 4,000 gallons per minute of sea water to the nanometer level at 99.9% efficiency. Physically filtering out viruses requires membranes and pressure and is only available on the desktop

scale.

Nor is there a series of systems that will eliminate viral contamination at this pace. The company may be loath to discuss this but is preparing for viral outbreaks in their mortality projections, I assure you. Most RAS systems growing salmon plan for a 22% loss between the egg and smoltification life stages. History shows that moralities from the smolt stage to the time the fish reaches the 4.5 kilos stage range from an absolute minimum of circa 10% to over 90% per cohort or stocking. An example would be the 33,000mt Grieg project being built in NL Canada. This project estimated pre smolt and extra large (1.5kgs) smolt losses at 22% despite using aquifer water and similar UV treatments of intake water that is only aquifer water. From their recently submitted EIS to the NL Canada Government:

Table 2.10. Egg importation schedule during ramp up (Years 2–5) and steady phase (Year 6 onward). Shipments in February will be used for seasonal productions.

Year	Order Month	Planned No. of Smolt to Sea	Extra to allow for mortality (%)	No. of Eggs Received
2	September	1,000,000	22	1,220,000
2	October	1,000,000	22	1,220,000
Total		**2,000,000**		**2,440,000**
3	June	1,000,000	22	1,220,000
3	August	1,000,000	22	1,220,000
3	October	1,000,000	22	1,220,000
Total		**3,000,000**		**3,660,000**
4	February	1,000,000	22	1,220,000
4	June	1,000,000	22	1,220,000
4	August	1,000,000	22	1,220,000
4	November	1,000,000	22	1,220,000
Total		**4,000,000**		**4,880,000**
5	February	1,000,000	22	1,220,000
5	June	2,000,000	22	2,440,000
5	August	2,000,000	22	2,440,000
5	November	1,000,000	22	1,220,000
Total		**6,000,000**		**7,320,000**
6	February	1,000,000	22	1,220,000
6	June	2,000,000	22	2,440,000
6	August	1,000,000	22	1,220,000
6	October	2,000,000	22	2,440,000
6	November	1,000,000	22	1,220,000
Total		**7,000,000**		**8,540,000**

[2] One degree-day is the mean temperature, above 0°C, experienced for a period of 24 h. For example, a salmon egg incubated at an average daily temperature of 10°C for 62 days, from fertilization to hatching, is said to have hatched in 620 degree-days.

Physical drum filters combined with membrane filters that reach down into the 0.4 micron range with >99% efficiency will help remove particles, but viruses in these larger none filtered particles will be heavily shielded from the extremely short treatment time (read millisecond aka instantaneous) of a UV blast. UV does not penetrate solids well if at all, Similarly, ozonation has limited effects when viruses are shielded in 0.4 micron and larger particles and treated for a very short duration like that used in the contact tank of the proponents design.

When Dr Fred Kibenge wanted to kill salmonid viruses in his experiments, he increased both the UV and exposure time to 1000s of time above what an industry sponsored study (Vike et al 2014) suggested is effective. He has done this for obvious reasons. He is the author of the benchmark book, Aquaculture Virology, (2016), ran one of only two OIE approved ISAv testing labs in the world, teaches at one of the most prestigious finfish vet universities in the world, and is the Editor of the peer reviewed scientific journal “Aquaculture”.

Given these facts, it is only a matter of time before every single virus that is in the ecosystem that the intake water is extracted from, enters the tanks. What happens when each of these viruses enters the bioreactor of a RAS system of this scale will be discussed below with some real world examples.

By far the best method of preventing viral contamination is to reduce to nearly zero the viable viruses in the intake water. Typically this is done by denaturing the virus structure with pressure and heat. This is not viable when millions of gallons a day are needed and so less effect methods are used like using virtually virus free aquifer water and extreme filtration and UVc. This is what virtually all RAS system are now doing globally. Virtually no hatcheries or major RAS project recently or being built are using surface water.

The list of bankruptcies or near bankruptcies is long and successes using surface water are zero. The famed Landsand Laks company in Denmark that is now behind the massive Atlantic Sapphire project in Miami (now projecting >200,000mt of production) used surface and seawater. The result was the near bankruptcy of the company, complete loss of several stocking cohorts, and nearly 18 months of shutdown to redesign the filtration system: https://www.hatcheryinternational.com/danish-salmon-ras-announces-temporary-shut-down-1599/ after 7 years of attempts, and embarrassing bacterial and virus nightmares while expanding into Miami, https://salmonbusiness.com/costly-moon-landing-for-land-based-salmon/ they are finally able to have a profit in 2019. Needless to say, after this experience, the new Miami project is not of a surface water extraction design.
“Johan Andreassen, the CEO of land-based salmon farmer Atlantic Sapphire, said the model in his US expansion would differ from the approach in Denmark, in order to protect the company from the risk of “unexpected mortalities”...” https://www.undercurrentnews.com/2017/07/07/land-based-salmon-farmer-hit-by-die-off-after-securing-funding-for-us-expansion/

Also of note, is that this is the industry leading company's track record. They are currently valued at nearly $1B USD and are yet to produce a single fish. https://www.intrafish.com/aquaculture/atlantic-sapphire-is-now-worth-800-million-really-/2-1-656420

Here is a partial list of major USA and global land based failures: https://www.intrafish.com/finance/analysis-heres-a-list-of-high-profile-land-based-aquaculture-failures/2-1-712748

All of this seems to be lost on Erik Heim who stated recently that he was not aware of many mass mortality events in RAS. Re: https://salmonbusiness.com/is-ras-tech-immature/

There are many reasons beyond excessive antibiotic use and fish mortality and health restricted growth rates and early smaller harvest sizes that NS Canada and many jurisdictions have banned the use of surface water in hatcheries. There are, according to various respected aquaculture project analysts like

Norwegian Development Bank, Rabobank, Parot, etc, more than 70 major land based aquaculture project that have been announced and are either in the planning or building stage. I can not think of one that uses surface water and has been approved and has started construction (Norway has two working on it though), but can name more than two dozen that are strictly using aquifer water and have started construction. Most RAS system that are now being planned or built today are designed around aquifer water for obvious reasons I will explore below.

Discrepancies Between Intake Water Treatment and RAS/Processing Plant Effluent :

1) drum filters are 10 micron on intake and 0.4 on effluent. If 0.4 is required to allow UVc penetration of the effluent it should also be required for the intake water.
2) Municipal water is simply carbon filtered without any micron filtration or UVc treatment, this assumes that town water treatment systems are design to remove and are monitored for fish pathogens. This is a serious design error.
3) 10 micron drum backwash water is dumped in the sewer from the concentrated pathogen laden sludge generated by the intake water (illegal by Maine law). This is collected and disposed of in the effluent treatment.

Brood Stock and Egg Import Issues :

In between intake water sources and effluent issues is another, very troubling source of repeated issues in the salmon aquaculture industry – disease importation and development in the brood stocks. This will be discussed more in depth below.

Every major brook stock supplier globally has had virus issues, including the proponent's supplier - Stofnfisker in Iceland. Chile banned this company in 2015 for producing VHSv infected fish. This virus crippled the entire Norwegian salmon industry for 2 years while shutting borders to their fish. Recently in 2019, they were caught shipping PRv infected fish to Washington for Cooke Aquaculture and 800,000 smolt had to be destroyed when government was alerted by eNGOs. In a recent interview with a leading trade magazine responding to the Washington bungle, the company spokesperson suggested that they sell both PRv infected fish and non PRv infected fish at a higher price. This does not bode well for their reputation in sustainability and transparency.

The proponent does not mention what strain of salmon will be used, but given that Stofnfisker sells Norwegian strain salmon, it is safe to assume the proponent wants, like virtually every salmon farmer in North America, to use this non-native strain. This will almost certainly introduce known non-native viruses to Maine, just as it did mere months ago in Washington. Unknown viruses, yet to be discovered like the many potential candidates that Dr Kristi Miller-Saunders is working on (per comm 2017), will of course also be transferred. There are very good reasons that this industry has been denied access to these fish and the Williamsburg Treaty exists that restricts companies to using local salmon as broodstock.

Certainly the proponent is in such a terrible rush while proceeding without any pilot testing of its design in the given location, thus risking the entire project, it will not take the time to develop St. John River strain broodstock as required by the Williamsburg Treaty, and used by all aquaculture facilities in

Maine currently ge Cooke Aquaculture. This is a Treaty that at all ICES members signed, including the USA, that states that local genetics will be used in all aquaculture developments for salmon. I suspect that EU smolt will be attempted to be brought in. This has never been done in North America despite industry trying for nearly 20 years. Every time this industry moved salmonids from the EU to North or South America they brought a host of viruses with them that to this day are causing serious harm to native fish stocks. Examples will be discussed below.

This serious risk (or imminent occurrence) of virus mutation in the bioreactor of a RAS system or introduction of non-native pathogens such as viruses via the use of non-native St. John River, or even more severe, the use of Norwegian strain salmon would be in contravention to the Maine State Environmental Protection Regulations which state:

Chapter 586: RULES PERTAINING TO DISCHARGES TO CLASS A WATERS

SUMMARY: This rule establishes criteria to define what constitutes effluent quality necessary to ensure the standards for class A waters are met.

1. Scope. Under 38 MRSA section 464 discharges to class A waters must be equal to or better than the receiving water in order to ensure that habitat, aquatic life, and bacteria are as naturally occurs. The following sections define effluent criteria necessary to ensure these requirements are met.

It could be argued that if granted a permit for this design, and importation of salmon, that government is setting the company up to break Maine's laws.

Effluent Treatment and Diversion or Shutdown Issues :

This next response to a public engagement question is also telling.

"*We are going to require you to have, in place, a plan to halt all circulation into the bay should a virus or disease outbreak in your tanks. Please explain in detail the steps that you would take.*

Pathogenic material will be unable to enter or leave our facility. We have extensive standard operating procedures (SOPs) for contingency situations at our European facilities. These SOPs, best practices, and biosecurity measures will be adapted and further expanded for our Belfast facility.

Our modules and tanks are separate entities and do not share water or materials from one module to the next. Materials and water from one module cannot and will not move from one module to another. This separation of modules provides an additional layer of biosecurity for the facility."

I would suggest, based on the design, that no ability to shut off the effluent for any extended period of time is available. Culling 10s of 1000s of fish from one or more tanks is a time consuming venture even if the equipment required is available immediately. Culling millions would be very difficult to get the proponent to do under any circumstances. They may pump air into the tanks but this is a guess as no intelligent response was given. There is no viable plan "b" as to where to put the effluent and I will suggest that effluent will continue to stream from the tank despite a diagnosed viral or other infection that bring pathogen titres to "biblical levels". Hence the list of antimicrobials and antiparasitics in the

list of chemicals that may/will be used if the pathogen is not viral. A viral issue could results in a mass die off and serious disposal and contagion issues. I have witnessed 3 industry wide meltdowns in NL since 2012 and can assure you that 7+ million dead rotting salmon of up to 10 pounds each is a serious logistical and environmental nightmare that requires mega infrastructure and planning. To date 17 million plus have died in NL since 2012.

The problem of fish effluent treatment, from processing or RAS plants, is an old one. After nearly 11 years of debate and research a team of EU vets finally produced a document that reviewed all the known available options. The result was that the only known financially viable and tested method for virus reduction to titres (aka dilutions) that were acceptable in terms of risks to wild fish would have to include: extreme filtration and burning of the solids, bleaching and de-bleaching of the effluent liquid (or evaporation and treatment of the solids – often deemed as too expensive), and then geo-filtration in a suitable clay based matrix. Needless to say, this did not go over well for processing plants dumping untreated effluent or hatcheries (Skall and Olesen 2014).

Unknown Viruses and or Hosts :

As suggested in world leading finfish virologist, Dr Fred Kibenge's benchmark 2016 book "Aquaculture Virology", we are only beginning to scratch the surface of finfish virology. This is evidenced by the large number of new viruses and new hosts for known viruses being found in aquaculture.

Perhaps an excerpt from the ICES Working Group on Pathology and Diseases of Marine Organisms published in 2018 may highlight just how primitive our current knowledge is by listing a number of serious disease issues and trends discovered that year:

"The group produced a report on new disease trends in the ICES area
based on national reports from fifteen member countries. **Notable reports for wild fish included observations of salmonid alphavirus SAV6 and Photobacterium damsellae in ballan wrasse, underscoring the need for disease surveillance in wrasse used as cleaner fish in salmonid aquaculture; the first observations of parasites Ichthyobodo salmonis and Desmozoon lepeophtherii in Pacific salmon from western Canada**; high prevalences of Loma branchialis and increasing prevalences of Contracaecum osculatum in Baltic cod from the eastern Baltic Sea; **and increasing prevalence of M74/thiamine deficiency in salmon yolk sac fry in Sweden. The considerable number of new and emerging disease trends in wild fish, all relevant to important fisheries, highlight the urgent need to continue disease monitoring of wild fish populations in the ICES region.**
Reports for farmed fish included the first cases of heart and skeletal muscle inflammation (HSMI) caused by piscine orthoreovirus (PRV) in Atlantic salmon in western Canada; the first report of infectious haematopoietic necrosis virus (IHNV) in Finland, in rainbow trout; the first report of I. salmonis in Atlantic salmon in Canada, in aquaculture in British Columbia; widening geographic distribution and increased effects on younger salmon of cardiomyopathy syndrome (CMS) caused by piscine myocarditis virus (PMCV) in Scotland and Ireland; and increasing cases of complex gill disease (CGD) in Atlantic salmon in Scotland. Complex gill disease is an increasing concern among ICES member coun-

tries, and better understanding and developing strategies to mitigate CGD will be a new focus of WGPDMO effort.“

It goes on to suggest that a pathogen screening list is still in development:
“ Work on additional documents included...a synthesis on amoebic gill disease in salmon, **and a compilation of pathogen screening in wild salmonids.**“

https://www.researchgate.net/publication/326467969_Report_of_the_Working_Group_on_Pathology_and_Diseases_of_Marine_Organisms_WGPDMO_13-17_February_2018_Riga_Latvia

Novel, but very serious disease are still unexplained and new viruses are being discovered at an alarming rate. For many known serious viruses we have no method to culture the virus to amplify it for testing; and for others, testing protocols are of unknown efficacy. Please review the presentation by Dr Kristi Miller-Saunders, she is the lead researcher of the world's largest salmonid virus hunting program, a >$10M CAD venture, based in Nainimo BC Canada at the Federal Department of Fisheries and Ocean lab and has been cited by more than 4,500 peer reviewed papers:
https://youtu.be/qfIGzDrTtJA?t=3011

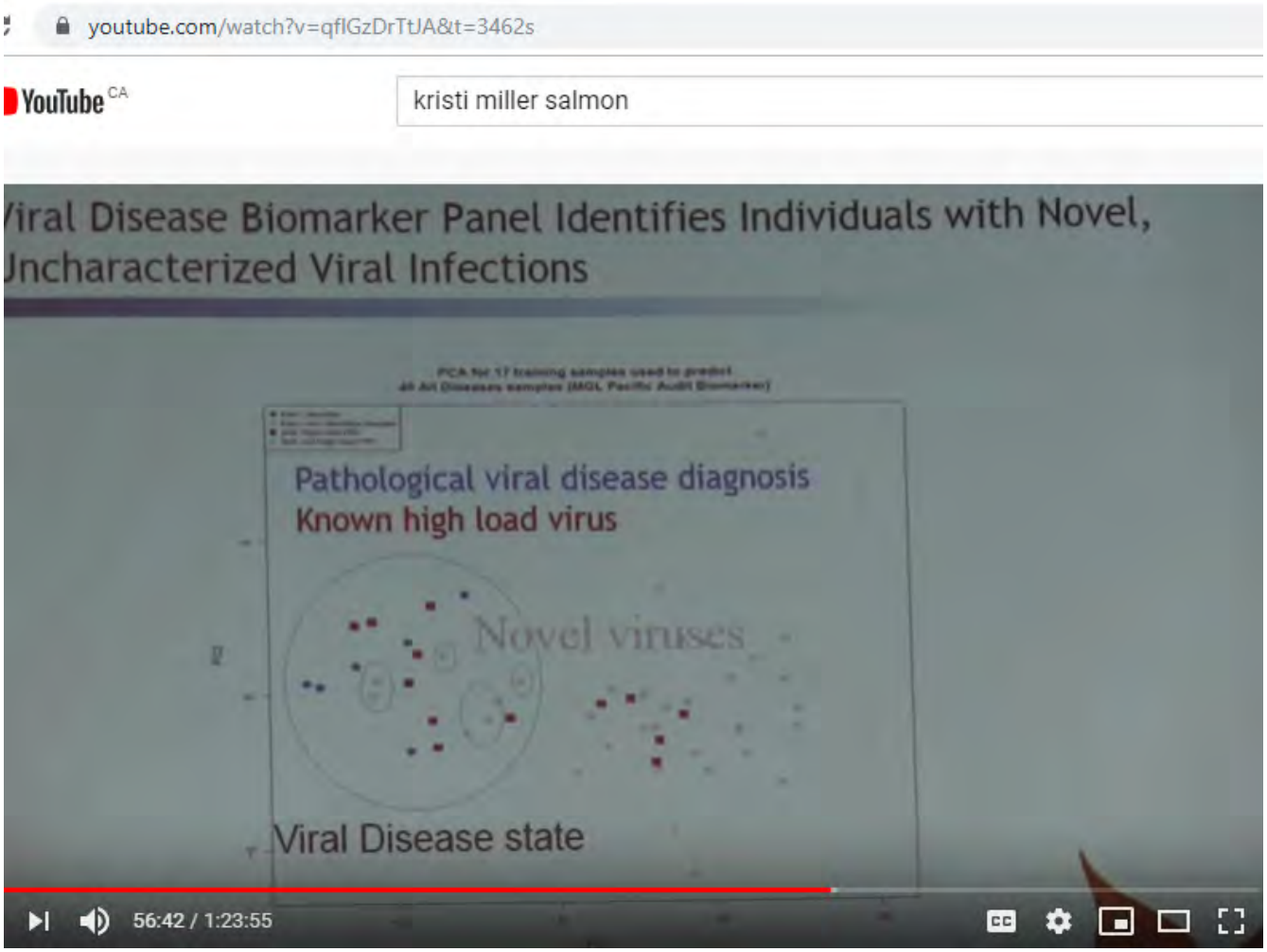
Of critical importance, when describing fish she had sampled recently, is the statement that the fish were in a diseased state (and shedding viruses) without showing any physical symptoms histologically. Often, the peak in virus shedding occurs prior to any physical symptoms.

This statement and situation is important as it suggests that any and all testing that any land based facility is doing, will not likely see or detect a pathogen until long after the disease is being amplified, possibly mutated, and expelled via the effluent. In some cases, depending on the virulence of the virus, this may be days, weeks, months, or even years. A “slightly sick” fish in a protected tank is not a big issue for the proponent, nor the government overseers, as 99% of fish diseases they are not transmissible to humans and thus able to be ignored without endangering public safety, or in many cases, the production of the fish in a tank. The same cannot be said for any wild fish that may become exposed to such effluent. This regulatory dichotomy between what is “good enough” for tanked fish grown for human consumption vs what is “good enough” for wild fish exposed to tank amplified and mutated contagions, will be discussed below.

Dr Kristi Miller-Saunder's presented some of her research in a 2017 presentation in BC. A graph from this presentation is seen showing new to science viruses.

The grey data points on the left of the graph showing fish in a diseased state (ie immunological responses) are all unknown viruses to science. This shows that a good percentage of all individuals in the samples in the graph are sick with unknown to science viruses.

What is also featured throughout her talk, is her use of “high put-through” genetic testing to examine the genes being expressed by the fish to show it is in an immunologically distraught state, ie sick with a triggered immune system. No aquaculture company, nor any USA regulatory agency is using this



cutting edge "high put through" qPCR gene screening for monitoring the health of RAS salmon. Nor do they screen fish or eggs using the methods used in Dr Millers lab. *Moreover, if indeed these methods were used, I think one would quickly see that, virtually no tank of Atlantic salmon held in captivity is comprised of even 50% "healthy" fish, after several months of being in that environment. The objective of a RAS system is not to mimic nature and produce fish fit to survive in nature and breed, but simply to get them to market. It has long been known that hatchery reared fish do not survive well in nature compared to naturally produced fish. Some of the reason this occurs is likely due to viruses in the broodstock intrinsic to the way the fish live and are artificially bred.*

Another telling statement of her talk is the fact that many viruses we know exists, due to genetic testing, science can not culture. This makes development of effective screening methods difficult or impossible as well as learning about vectoring between species, mode of pathology, etymology, (Kibenge 2016, Miller 2017 personal communication – see video).

A single virus in a single egg or broodstock fish is likely to be quiet prevalent in the population after some months in a tank environment. Predators, struggles in environmental challenges, struggles in migrating fast flowing rivers with rapids, waterfalls, etc will ensure that only the fit survive. This is the complete opposite of a stable tank environment where viruses of low and medium pathology can and do flourish. **A mortality rate of 22% is common in RAS hatcheries before the fish reach the smolting stage (circa 2-3oz and 6 to 8 inches)** *but very often only a tiny percent, if any at all, of these*

mortalities can be definitively diagnosed to a single or multiple known pathogens. We simply do not know what we are doing regarding fish culturing.

Moreover, studies show that for many viruses, the peak in viral particle shedding occurs before the fish become physically sick, exhibits changes in behaviour, or physical histological symptoms. By the time any of these visually detectable conditions are manifested the fish's immune system has started to reduce the virus particle production and shedding. Ie It is too late in terms of prevention of viral management of mutation of the virus, or escape via the effluent. This is the “perfect storm” that Dr Fred Kibenge discusses in the preface of his benchmark book, Aquaculture Virology. Dr Kibenge is a professor in one of only two Universities in Canada that all our aquaculture fish vets are trained in. He is also the editor of the peer reviewed prestigious journal “Aquaculture”.

Regarding the 7 grey data points on the left of Dr Miller-Saunders graph at time 56-57 minutes, she and her colleagues recently published Mordecai et al 2019 which is turning the salmon farming industry on it's head: https://elifesciences.org/articles/47615

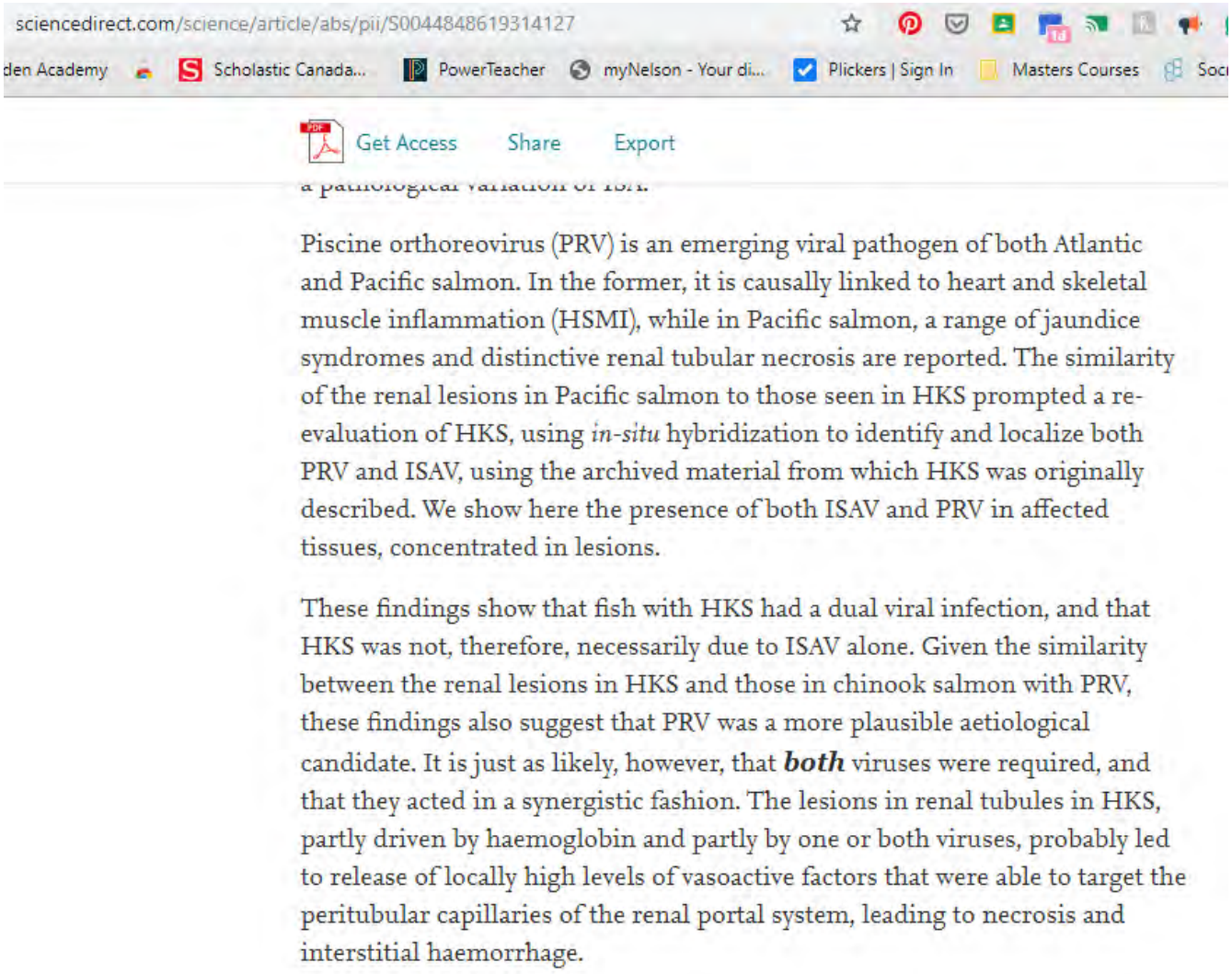
Her team has discovered three new viruses among now decimated and endangered Chinook and sockeye salmon populations. One of the viruses belongs to a group not known to infect fish and thought to have a completely different infection strategy, more similar to its closely related viruses that infect mammals.
In total, scientists sampled DNA from 6,000 salmon. The new viruses were found in hundreds of dead and dying farmed salmon, as well as in wild specimens from stocks that have inexplicably collapsed recently.
"It emphasizes the potential role that viral disease may play in the population dynamics of wild fish stocks, and the threat that these viruses may pose to aquaculture," said UBC virologist Curtis Suttle in an interview by his University.

One of the three new viruses was identified in more than 15 percent of all the tested hatchery Chinook salmon. Another was found present in 20 percent of farmed Chinook.

While such a discovery may seem alarming, and scientists are pointing to this as an example of likely causes to the decline of dozens of stocks of pacific salmon, Atlantic salmon, and trout stocks, it is not unusual or even irregular.

New to science highly pathogenic viruses are being discovered annually. Many of the world's top finfish virologist's think that aquaculture may be a primary incubation point for virus mutation.
Recent examples abound but would include:
PMCv
SAv6
HKS caused by previously thought benign PRv and ISAv together
etc eg

See Ferguson et al 2019 (screenshot above) for an example of brand new to science viral interactions that have been ignored by industry for decades yet are now known to cause severe issues in wild fish. PRv is ignored in Maine. Most hatchery/aquaculture salmon test positive (Morton Unpublished data, personal communication)



Moreover, via the protective “bioreactor” aquaculture provides, new strains are discovered monthly and even weekly. Again, many of the world's top finfish virologists think that aquaculture may be a primary incubation point for virus mutation. Examples would include the 106 new to science strains of ISAv found in salmon open net pens in the NW Atlantic since 2012 as seen here: https://www.inspection.gc.ca/animals/aquatic-animals/diseases/reportable-diseases/isa/locations-infected/eng/1549521878704/1549521878969

For decades industry vets proclaimed that viruses like ISAv could not be transmitted from broodstock to eggs. Once this is proven, it should mean eradication of extremely valuable (100s of millions of USD) broodstock that took decades to develop. Thus industry was determined to make sure that didn't happen. A fight among scientists and vets ensued in peer reviewed journals with industry funding PhDs and Universities and NGOs funding PhDs. Now, today, beyond any question some of the viruses, like ISAv and others, are known to be transmissible to offspring by the parents.

One would think that the industry would then be required to cull the brood stock of these viruses. Instead, nothing has been done. The issue is how healthy must a fish be, if the goal is to get it to market regardless if it carries fish pathogens that are not harmful to humans and not regulated.

Many papers have been published (eg Nylund et al 2007) for more than a decade suggesting that ISAv mutation from a none mortality inducing strain/type called HPR0 in an aquaculture setting ie “fish flu” to a virulent mortality causing strain called HPR-deletion (types 1-6) is occurring in the “bioreactors” used by salmon aquaculture. Proving this and catching the virus “in the act” so to speak is almost impossible. In fact, only recently were scientists able to prove such an event in a net pen whereby a non pathogenic strain of ISA called ISA HPR0 mutated to a more pathogenic form.

Issues with Vectors/Hosts, Transmission, Monitoring, Motivation, and Science :

No complete list of all known viruses have been tested for in the fish present in the intake water supply. Thus, government, nor the proponent has any baseline as to what is present before the project starts.

It also leaves everyone guessing where to focus limited monitoring and testing efforts.

A common criticism of regions with intensive aquaculture is that **not enough money is charge for fees to allow sufficient monitoring for effects.** Norway has tabled a tax that amounts to 62% that is expected to pass. This is a 40% increase. Moreover, compared to Maine, Norway charges 17,000 to 36,000 CAD per mt of fish produced via a licence, Maine charges less than $1 CAD/mt in fees and licenses.

Similarly, science is still piecing together thee most basic understanding of transmission vectors for ANY of the known salmonid and finfish viruses in general, Kibenge 2016. Even some of thee most screened for viruses like ISAv are transmitted from one fish to another or one species to another by unknown means and vectors, McBeath et al 2014. Kibenge et al 2016 found a strain of ISAv in BC that had a mutation in the screening primer section of the virus that made detection using standard techniques impossible.
Industry has limited time and money to focus on virus detection. As such viruses known to cause mass mortality are the only focus of genetic testing. For example, in Canada, the Authority Responsible, the Canadian Food Inspection Agency, not only has no mandate to protect wild fish, it only lists 5 viruses that must be reported if found. These are the 5 listed by the OIE (the United Nations Vet Board responsible for limiting disease spread that may seriously threaten our traded food supply). Ie CFIA lists the bare minimum of protection and then only for our financially significant food supply. Maine does the same.

Even screening of such OIE listed viruses is limited. It is not an issue until it is a serious issue in the tanks. For example. 5 fish every 30-45 days from a tank holding 50,000 fish is a typical genetic screening protocol for even the most deadly of the 5 OIE reportable viruses like ISAv, VHSv, SAv, IPNv, etc. Others like PMCv, PRv ENv, etc are ignored.

Viruses that can be carried by salmon in a RAS system yet do not cause serious financial losses are ignored, especially if the primary host is a different fish species. So while a striped bass virus, for example, might be carried by the salmon without pathological symptoms in the salmon, it would be ignored by monitoring and thus the facility would act as an unnatural year around reservoir that kills striped bass. The vets would simply shrug and suggest, we don't know what is causing it and “we have no evidence” to suggest that aquaculture is to blame. Finfish vets are only employed by two groups:

industry and government departments that rely on industry to do well. Neither have a financial benefit from protecting wild fish.

The principle and secondary reservoir hosts are also not known with new ones constantly being discovered for ALL of the known viruses at an alarming rate eg Di Cicco et al 2018. Virtually every study done results in a lengthening of the list of finfish that can carry and transmit the virus. The same is true for fungal, bacterial and parasite carriers of finfish viruses. Once an amplified contagion leaves the RAS facility – whether it affected the health of the salmon or not, we have no idea what will happen beyond the end of the pipe.

Unintentional Dramatic Virus and Parasite Introductions :

What might be even more alarming is how often and how deadly the aquaculture industry is at transferring pathogens from one biologically isolated region to another via eggs and fish shipments. Examples might include the destruction of 41 salmon rivers in Norway by a skin parasite transferred from a hatchery in Sweden. The river have never recovered even today, nearly 40 years later. A more recent example is the very likely transfer via rainbow trout of VHSv 4a from the Pacific to the NW Atlantic before April 2016 in NL. This never before seen Pacific virus then ravaged through the herring stocks from April 2016 through 2017 through 2017 all along the NW Atlantic ocean. Immediately after, and for the first time in NL history, the entire spring purse seine herring fishery was shut down completely. Occurrences like this, while almost impossible to prove, abound. Another recent 2017/18 example was the amplification, mutation and escape of VHSv in salmonid hatcheries and aquaculture facilities in Alberta. This spread into and throughout local rivers decimating fish stocks and causing unknown ecological cascade effects. In spring 2016 VHSv 4a strain (pacific only virus) was found in herring near NL salmonid net pens. This primarily rainbow trout disease has following aquaculture around globally and is now lose in the Atlantic Ocean. It spread through herring stocks throughout the NW Atlantic during a winter of mass die offs - 2016/17. That spring the NL south coast purse seine herring fishery was shut down for the first time in NL history. It was all over the media. The aquaculture industry claims it was not them. I will not belabour the point but will list a few more recent examples below with concrete evidence.

Viruses in Broodstock and Hatcheries in NE North America and Beyond :

Many known and likely unknown viruses are found in the broodstock of virtually every company. Every year many hatcheries get caught selling virus infected eggs, fry, or smolt. This often includes the proponent's chosen hatchery.

Dartek Nova Scotia. 2017. This hatchery was selling ISAv infected fish and got caught.

Despite this, the salmon sold by this company were stocked in NL - later several massive ISAv outbreaks were detected and the fish were gradually culled or died. The sites held roughly 850,000

Infectious Salmon Anemia reported in land-based aquaculture facilities

Fisheries and Aquaculture
March 2, 2018 9:30 AM

The presence of Infectious Salmon Anemia (ISA) at two land-based aquaculture facilities in Nova Scotia was confirmed in February.

The virus must be reported under federal regulations and the province notified the Canadian Food Inspection Agency (CFIA).

The sites were quarantined in early February once initial tests indicated the suspected cause of infection was the virus. The affected fish have been removed and disposed of in a safe and secure manner.

The virus poses no risk to humans. In these two land-based cases, the risk of the disease spreading to the wild population is considered to be very low to none at all.

Infectious Salmon Anemia has been present in Atlantic Canada since 1996. The disease-causing version of the virus has not been detected in Nova Scotia since 2012.

salmon each when initially stocked. Millions of fish died or were culled. https://www.mae.gov.nl.ca/env_assessment/projects/Y2018/1975/1975%20Appeal%20document%202018%2011%2005.pdf

VHSv Iceland 2015

Caught harbouring and amplifying VHSv infected fish in a supposed biosecure egg production facility used by clients globally. This is the same company that the proponent wants to use to import non-native salmon carrying heaven knows what.

PRv from the same hatchery in Iceland caught by Washington in 2018. https://wdfw.wa.gov/news/wdfw-denies-permit-company-place-800000-atlantic-salmon-puget-sound-net-pens

World News

Search by date - Go Search by country All

Salmon eggs. (Photo: Stock File)

Salmon egg import from Iceland suspended

CHILE
Tuesday, October 27, 2015, 22:00 (GMT + 9)

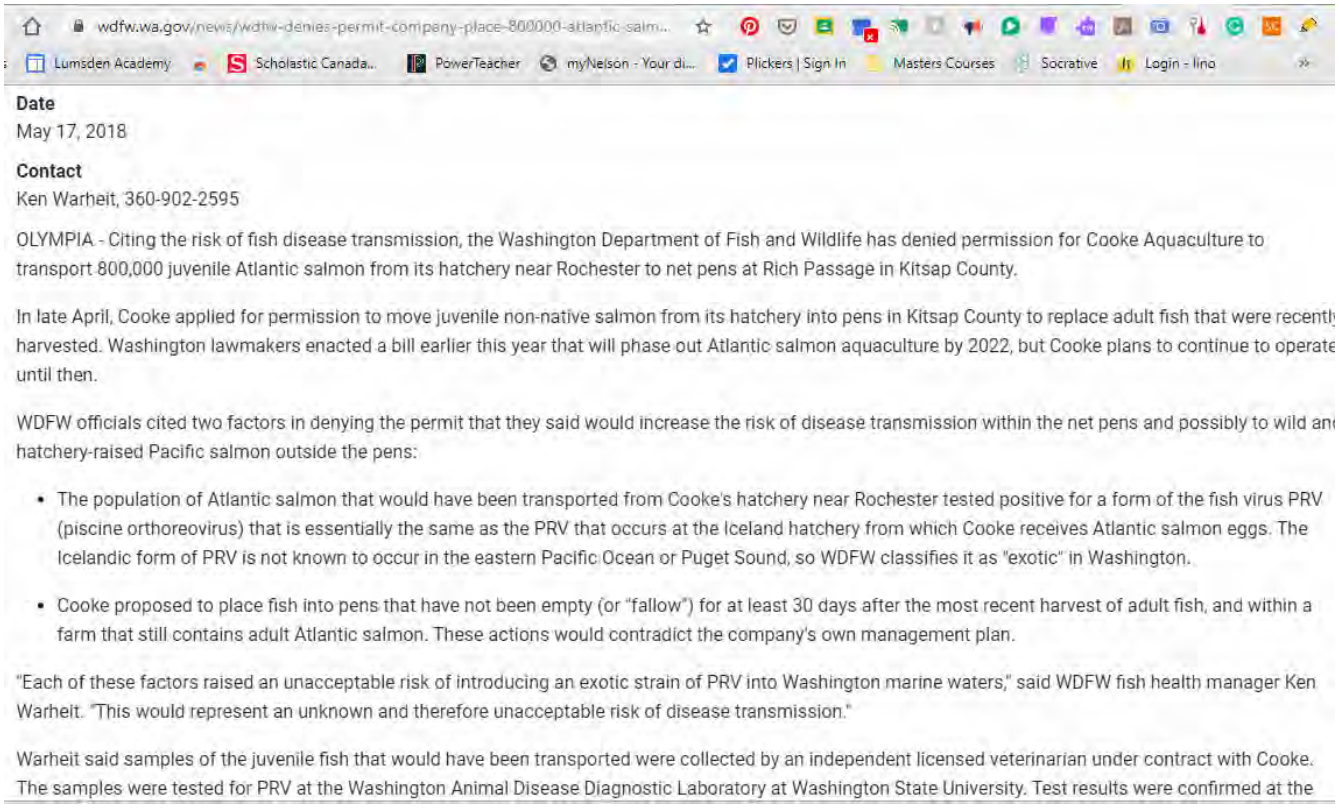
The National Fisheries and Aquaculture Service (SERNAPESCA) decided to suspend Atlantic salmon eggs imported from Iceland, given the health alert issued by the World Organisation for Animal Health (OIE) generated by the notification of the occurrence of viral hemorrhagic septicemia (VHS) virus in that country.

The measure to suspend imports was provided in Resolution 9844 of the agency, under the Supreme Decree 72. This regulation, which establishes the health requirements for import, in its Article 10 the suspension to carry out a new health assessment is considered, given the significant changes in the epidemiological situation of the country of origin.

SERNAPESCA emphasizes that the first detection of this disease is confirmed in Iceland, wild fish used as fish cleaners, and the case is still under investigation.

editorial@fis.com
www.fis.com

The Washington import of PRv was from the same Icelandic company StofnFiskur (same as the VHSv issue in Chile cited above) that the proponent stated would be used. The company admitted to harbouring broodstock infected with PRv and charging more for eggs that were free of PRv. None of the regulatory agencies caught this ongoing multi-year issue until an NGO forced the issues in Washington. https://salmonbusiness.com/egg-supplier-responds-to-washington-prv-salmon-cull/



wdfw.wa.gov/news/wdfw-denies-permit-company-place-800000-atlantic-salm...

Lumsden Academy | Scholastic Canada... | PowerTeacher | myNelson - Your di... | Plickers | Sign In | Masters Courses | Socrative | Login - lino

Date
May 17, 2018

Contact
Ken Warheit, 360-902-2595

OLYMPIA - Citing the risk of fish disease transmission, the Washington Department of Fish and Wildlife has denied permission for Cooke Aquaculture to transport 800,000 juvenile Atlantic salmon from its hatchery near Rochester to net pens at Rich Passage in Kitsap County.

In late April, Cooke applied for permission to move juvenile non-native salmon from its hatchery into pens in Kitsap County to replace adult fish that were recently harvested. Washington lawmakers enacted a bill earlier this year that will phase out Atlantic salmon aquaculture by 2022, but Cooke plans to continue to operate until then.

WDFW officials cited two factors in denying the permit that they said would increase the risk of disease transmission within the net pens and possibly to wild an hatchery-raised Pacific salmon outside the pens:

- The population of Atlantic salmon that would have been transported from Cooke's hatchery near Rochester tested positive for a form of the fish virus PRV (piscine orthoreovirus) that is essentially the same as the PRV that occurs at the Iceland hatchery from which Cooke receives Atlantic salmon eggs. The Icelandic form of PRV is not known to occur in the eastern Pacific Ocean or Puget Sound, so WDFW classifies it as "exotic" in Washington.
- Cooke proposed to place fish into pens that have not been empty (or "fallow") for at least 30 days after the most recent harvest of adult fish, and within a farm that still contains adult Atlantic salmon. These actions would contradict the company's own management plan.

"Each of these factors raised an unacceptable risk of introducing an exotic strain of PRV into Washington marine waters," said WDFW fish health manager Ken Warheit. "This would represent an unknown and therefore unacceptable risk of disease transmission."

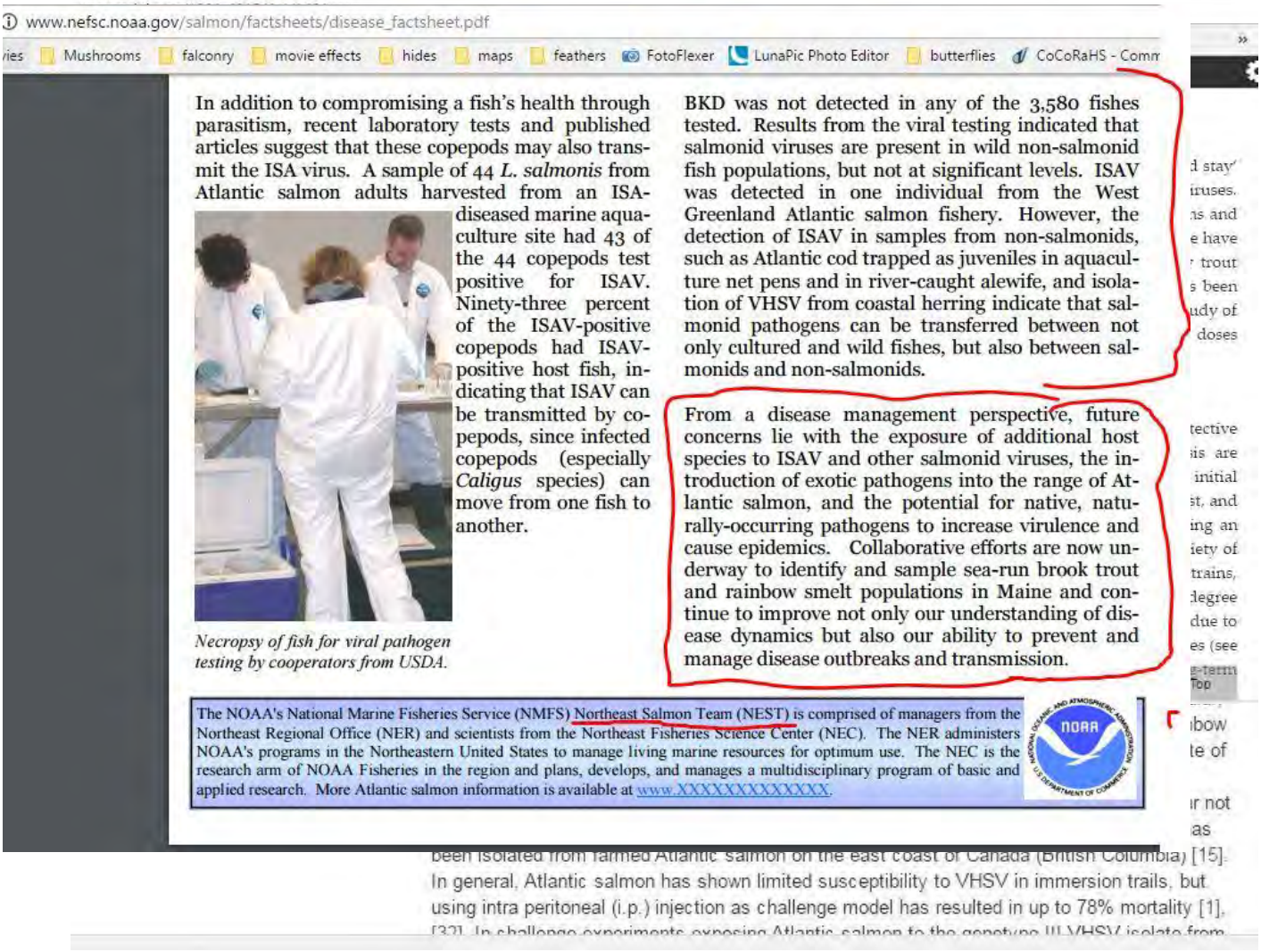
Warheit said samples of the juvenile fish that would have been transported were collected by an independent licensed veterinarian under contract with Cooke. The samples were tested for PRV at the Washington Animal Disease Diagnostic Laboratory at Washington State University. Test results were confirmed at the

New strains, “species”, and pathologies are constantly being found. Hugely important and basic questions about most finfish viruses remain today for our most studied viruses. Two examples are below.

Above from Purcell et al 2012

NOAA admits there are serious and basic information gaps.

ISAv HPR0 Marine Harvest was caught breeding eggs, fry and smolt with ISAv HPR0 in its Stephenville NL Canada close containment strictly aquifer supplied biosecure RAS facility in 2018. Later, marine sites stocked with these fish experienced mass mortality when the ocean warmed to typical peak summer temperatures. More than 3 million net pen fish died. Months later, when pushed



www.nefsc.noaa.gov/salmon/factsheets/disease_factsheet.pdf

Mushrooms falconry movie effects hides maps feathers FotoFlexer LunaPic Photo Editor butterflies CoCoRaHS - Comm

In addition to compromising a fish's health through parasitism, recent laboratory tests and published articles suggest that these copepods may also transmit the ISA virus. A sample of 44 *L. salmonis* from Atlantic salmon adults harvested from an ISA-diseased marine aquaculture site had 43 of the 44 copepods test positive for ISAV. Ninety-three percent of the ISAV-positive copepods had ISAV-positive host fish, indicating that ISAV can be transmitted by copepods, since infected copepods (especially *Caligus* species) can move from one fish to another.

Necropsy of fish for viral pathogen testing by cooperators from USDA.

BKD was not detected in any of the 3,580 fishes tested. Results from the viral testing indicated that salmonid viruses are present in wild non-salmonid fish populations, but not at significant levels. ISAV was detected in one individual from the West Greenland Atlantic salmon fishery. However, the detection of ISAV in samples from non-salmonids, such as Atlantic cod trapped as juveniles in aquaculture net pens and in river-caught alewife, and isolation of VHSV from coastal herring indicate that salmonid pathogens can be transferred between not only cultured and wild fishes, but also between salmonids and non-salmonids.

From a disease management perspective, future concerns lie with the exposure of additional host species to ISAV and other salmonid viruses, the introduction of exotic pathogens into the range of Atlantic salmon, and the potential for native, naturally-occurring pathogens to increase virulence and cause epidemics. Collaborative efforts are now underway to identify and sample sea-run brook trout and rainbow smelt populations in Maine and continue to improve not only our understanding of disease dynamics but also our ability to prevent and manage disease outbreaks and transmission.

The NOAA's National Marine Fisheries Service (NMFS) Northeast Salmon Team (NEST) is comprised of managers from the Northeast Regional Office (NER) and scientists from the Northeast Fisheries Science Center (NEC). The NER administers NOAA's programs in the Northeastern United States to manage living marine resources for optimum use. The NEC is the research arm of NOAA Fisheries in the region and plans, develops, and manages a multidisciplinary program of basic and applied research. More Atlantic salmon information is available at www.XXXXXXXXXXXXX.

NOAA

been isolated from farmed Atlantic salmon on the east coast of Canada (British Columbia) [15]. In general, Atlantic salmon has shown limited susceptibility to VHSV in immersion trails, but using intra peritoneal (i.p.) injection as challenge model has resulted in up to 78% mortality [1], [32]. In challenge experiments exposing Atlantic salmon to the genotype III VHSV isolate from

by the media thanks to whistleblowers, the company was forced by the government to admit ISAv HPR deletion (a mutated deadly strain) was detected at three of the sites after never mentioning it during the die off despite knowing about it for 3 months prior.

In fact, many ?most? of thee top salmonid virologists in the world like Dr Are Nylund, Dr Kristi Miller-Saunders, and thee top government vets in Norway have all been warning the industry that they simply must take the financial hit and clean up their brood stock as it pertains to known harmful viruses and viruses thought or proven to mutate into virulent form in a tank or net pen:

Despite this, virtually no studies exist examining the differences between local wild salmonid and hatchery reared salmonids. The one study that does exist shows massive infection rates in all 13 of the critical government hatcheries in BC Canada as well as seasonal fluctuations and between site differences (Nekouei et al 2019). This is despite a variety of surface and subsurface water usage and state of the art treatment in many cases.

salmonbusiness.com/researcher-warns-of-isa-impact-on-trade/

Tissue samples showed the virus was identical with those in Norwegian aquaculture. Nylund concluded that a Norwegian brood-stock firm had send infected eggs to Chile.

"The virus had moved from Norway to Chile. You might want to believe that God put two identical viruses in two places, but research doesn't operate with God," Nylund asserted.

The price to pay

Nylund said he's documented that the virus spreads down through generations, as opposed to the popular belief that the virus is transferred "sideways". The discovery could lead to a solution, Nylund said, adding that the industry itself needs to seize the opportunity.

"This is an opportunity to do something about it, by going in and screening for the virus in the brood fish. The potential for infection from Norwegian fish should be minute," Nylund said.

"The industry has the opportunity, here, to go in and work with brood fish. We need to ensure the brood is free of infection. It's cost the industry must incur."

and become the ISA virus. HPR0 is found in most hatcheries," he noted.

"We also know that different environmental conditions can cause the virus to mutate."

In addition, if several of the RAS facilities are not shut off, fallowed and disinfected, the virus can build up.

"Land-based facilities can be a part of the future -- they have some advantages, but the technological solutions must not override important infection-hygienic principles," Rønningen said.

Perhaps this lack of monitoring is not surprising, given that the same vets and government departments that run these stocking hatcheries would be culpable for any impacts they caused on wild stocks. What is known, is that hatchery or RAS produced fish generally do not survive well in the wild. The single study done to date examined Pacific salmon and focused on diversity and viral load rather than potential effects from individual viruses, ignored surface water effects on the pathogens in a hatchery, ignored differences in individual pathogens, and seems designed to support the continuation of hatchery based multimillion dollar wild fisheries such as those in BC and Alaska where river stocking is big business (Nekouei 2019). It was also funded and designed by these same agencies, and in many cases individuals, that would be held responsible for the demise of the wild stocks had a link between the hatcheries and the demise of sympatric wild stock been found.

Ironically, despite a conclusion of - its not our fault-, the authors did suggest that: " There is a clear knowledge gap regarding pathogens that can adversely affect the performance and survival of Coho salmon. " The same is true for Atlantic Salmon.

Similar behaviour can be seen by culpable regulatory agencies like Canada's DFO when publishing peer reviewed papers that require full disclosure of funding by a potentially biasing source - yet simply not reporting it: eg https://watershed-watch.org/Scientific_Reports_Polinski_et_al_2019.pdf

In fact, the Cohen Commission uncovered a hornets nest of cover-up, not supplying required papers, emails, and data, that resulted in the lead Government Vet, one Dr Marty, facing a Federal Supreme Court challenge to his practices which was later withdrawn after the Vet Board (whom he helped lead) agreed to investigate his practices.

These are must watch if you think this industry and those managing it are trustworthy beyond being profiteers and industry shills in many cases:

Canada's Commission for Environment and Sustainability audited this industry and suggested it was the most disturbing audit she had ever conducted: https://www.youtube.com/watch?v=f2JZev5oYZ4

The Canadian DFO's own lead salmon virus hunter Dr Kristi Miller Saunders went public about cover-ups within government and regulatory capture: https://www.youtube.com/watch?v=VSIZgWdW0T8

This shocking documentary was a result of the Cohen Commission inquiry into the collapse of BC wild salmon:
https://www.youtube.com/watch?v=fTCQ2IA_Zss

A national TV channel in France produced a documentary which is revealing in several regards and should be viewed:
https://www.youtube.com/watch?v=MgrFXN4d1Jc

https://thetyee.ca/News/2018/01/10/DFO-Deadly-Farmed-Salmon-Disease-Downplay/

Despite strong evidence of similar practices in Atlantic Canada concerning at least two ISAv outbreaks and a VHSv outbreak, the public has been denied access to samples and reports that would incriminate the vets involved. They simply refused to supply the information and samples. Often this is done under the guise of “Third Party Harm” redaction and refusals.

So, even if the public in Maine catch the company, company vets, government vets, etc that are culpable will be in charge of the evidence. Even if a formal inquiry is called to investigate the incident, the public is likely to find they will simply not supply the rope to hang them with.

When asked if the public would have access to take their own samples for testing, the company avoided the question and suggested they would talk to groups about it. See
“*Will you allow the public to view or conduct sampling?*
Sampling will be conducted as required by any final permit in accordance with specific protocols outlined in said permit. We have been contacted by groups with a documented science and/or environmental background that are interested in assisting with this sampling, and Nordic will discuss such future cooperative sampling opportunities.”

The following is an except from the **2016 report by the ICES Report of the Working Group on Pathology and Diseases of Marine Organisms**. I include a large section from the wild fish and farmed fish in an attempt to show just how prevalent disease is in hatchery, how often new diseases follow the aquaculture industry around the globe, how the broodstock and eggs can often not be disinfected by treatments, and finally how often new to science disease are being found. Please remember, a sick wild fish is unlikely to breed. All of the sick wild fish tested were near aquaculture facilities.

Wild Fish
Viruses
Salmon gill poxvirus (SGPV) – Reported for the first time in Canada from **a healthy** adult Atlantic salmon in the Magaguadavic River, New Brunswick (a hatchery stocked river). **The finding was based on cytopathology and high-throughput DNA sequencing.**

Piscine reovirus (PRV) – Reported from Denmark in 2014 for the first time, 6% of 176 Atlantic salmon brood-stock tested positive by qPCR. **The virus was later detected in progeny (fry) from the affected brood fish despite disinfection of eggs. Eight wild brown trout were found to be negative for the virus.**

Infectious pancreatic necrosis virus (IPNV) – In mid-Norway, the virus was detected in gill samples in 7 of 670 of returning Atlantic salmon in four rivers in 2013 and 2014.

Infectious salmon anaemia virus (ISAV) – In mid-Norway, the virus (HPR0) was detected in gill samples in 16 of 670 of returning Atlantic salmon in four rivers in 2013 and 2014, and in 2014, the virus was also detected in 5 of 204 Atlantic salmon and 2 of 18 sea trout caught in marine estuaries in the same region.

Viral haemorrhagic septicaemia virus (VHSV) – A rare observation of Genotype 1b was made in a Baltic cod from Hanö Bay, Sweden (ICES district SD 25). The fish also showed signs of fin rot, purulent exudate, splenic granulomas, endo- and pericarditis, anaemia and peritoneal haemoraging...

5.1.2 Farmed Fish Viruses
Infectious pancreatic necrosis virus (IPNV) – In Sweden, two cases of IPN serotype ab were diagnosed in rainbow trout in a national screening program. One of the farms was in the Baltic Sea, the other in an inland lake. In Norway, the number of cases declined from 48 in 2014 to 30 in 2015, continuing a trend reported previously.
IPNV was found in Atlantic halibut **fry** in Norway on two occasions.

Infectious salmon anaemia virus (ISAV) – The disease was diagnosed in 15 Atlantic salmon farms in Norway, an increase from 10 farms in each of the two previous years. **Only three cases were considered primary outbreaks, one in brood fish, one at a sea site, and the third in a smolt farm.**

Four secondary cases received fish from the smolt farm. The remaining secondary cases were likely caused by horizontal spread from neighboring farms. Two epidemics in northern Norway from 2013 and 2014 are still not declared eradicated. At two sites, rainbow trout were infected following infection of Atlantic salmon at the same site. These cases are the first registered in rainbow trout under ordinary farming conditions. **In eastern Canada, sporadic outbreaks with the North American genotype persist, however surveillance revealed a high prevalence of European type HPR0 strains.** In western Canada, 0 of 2207 Atlantic salmon tested positive by qRT-PCR.

Salmonid alphavirus (SAV) – In Norway, there are two endemic regions with two subtypes of the virus, SAV2 and SAV3, and the northernmost part of the country is surveilled to maintain SAV-free status. One case of SAV2 was seen in Atlantic salmon in this region, and the affected population was immediately culled. During 2014 and 2015, there have been cases of pancreas disease (PD) caused by SAV2 in the SAV3-zone. The number of PD cases in 2015 was 135, close to the historically high number of 142 in 2014. Ireland experienced seven outbreaks of PD, after only three in 2014.

Piscine orthoreovirus (PRV) – Heart and skeletal muscle inflammation (HSMI) was diagnosed for the first time in Ireland at one marine Atlantic salmon site, and detection of PRV was confirmed by qPCR. Mortality was reported to be low. In Norway, the number of HSMI outbreaks in Atlantic salmon was 135, a reduction from the historical peak of 181 in 2014 that coincided with national delisting of this disease. Using qRT-PCR, **the virus was detected for the first time in eastern Canada in all Atlantic salmon from one lot held in quarantine**. **In addition, 6 of 11 salmon originating from another hatchery and held at a government research facility tested positive**. None of the Canadian salmon were examined histologically for evidence of HSMI. Onchorhynchus mykiss reovirus – **A new viral disease in rainbow trout first reported in 2013 from four different hatcheries in Norway was documented in the WGPDMO report from 2015. This disease had also caused mortality in fish transferred to seawater. Sequencing of the new viral agent showed that it is related to PRV in Atlantic salmon. No disease outbreaks have been registered in 2015, however, the virus was detected at 9 marine sites from among 50 farms tested**.

Salmon gill poxvirus (SGPV) – Salmon gill poxvirus disease has been known in Norway **since 1995**. The first genome sequence of this DNA-virus was described in **2015**. SGPV was diagnosed in a total of 18 Atlantic salmon farms last year, 15 marine sites and **three smolt farms**.

Bacteria Aeromonas salmonicida – One case was diagnosed in a marine Atlantic salmon site in Ireland. In Norway, A. salmonicida subsp. salmonicida was isolated in one case of increased mortalities in lumpfish transferred to a sea-site containing vaccinated Atlantic salmon, which were not affected. In Scotland, atypical A. salmonicida has been detected in moribund ballan wrasse being used as cleaner fish for farmed Atlantic salmon. In Norway, atypical A. salmonicida has been diagnosed in lumpfish used as cleaner fish in 51 cases, and in wrasses in 32 cases. Atypical A. salmonicida was found in three cases in Norway, all involving Atlantic halibut fry.
Yersinia ruckeri – Norway had 34 cases of yersiniosis in Atlantic salmon in 2015, eight in smolt farms, 25 in sea farms, and one in brood fish. Detected cases have increased over the last four to five years. As a consequence, smolt farms are increasingly using vaccines.
Moritella viscosa/winter ulcers – Winter ulcer syndrome was diagnosed in Atlantic salmon from three sites in Scotland and three sites in Ireland. In Norway, 57 cases of winter ulcers in Atlantic salmon and

four cases in rainbow trout were diagnosed, compared to 44 cases in salmonids in 2014. Vibrio-infections – In Norway, Vibrio anguillarum has been isolated from diseased cleaner fish used to control salmon lice. The bacterium was detected in lumpfish from twelve farms and wrasses from two. Three cases of V. ordalii have been reported in lumpfish. Flavobacterium/Flexibacter – Three cases of infection with Flavobacterium psychrophilum in rainbow trout were reported in Norway, two from marine sites and the third from an inland farm. Two cases were registered in 2014, and septicemic flavobacteriosis in rainbow trout has been a list 3 disease in Norway since that time. Pasteurella/ Pseudomonas – In lumpfish from Norway, Pasteurella sp. was isolated in 14 cases and Pseudomonas anguilliseptica in four. Piscirickettsia salmonis – The range of salmonid rickettsial septicaemia in Atlantic salmon in western Canada expanded to a new management zone and the disease now occurs throughout the year in some locations. Between 2013 and 2015, the annual number of diagnoses in Atlantic salmon has increased from 8.5% to 29% and in Pacific salmon, from 4% to 38%.

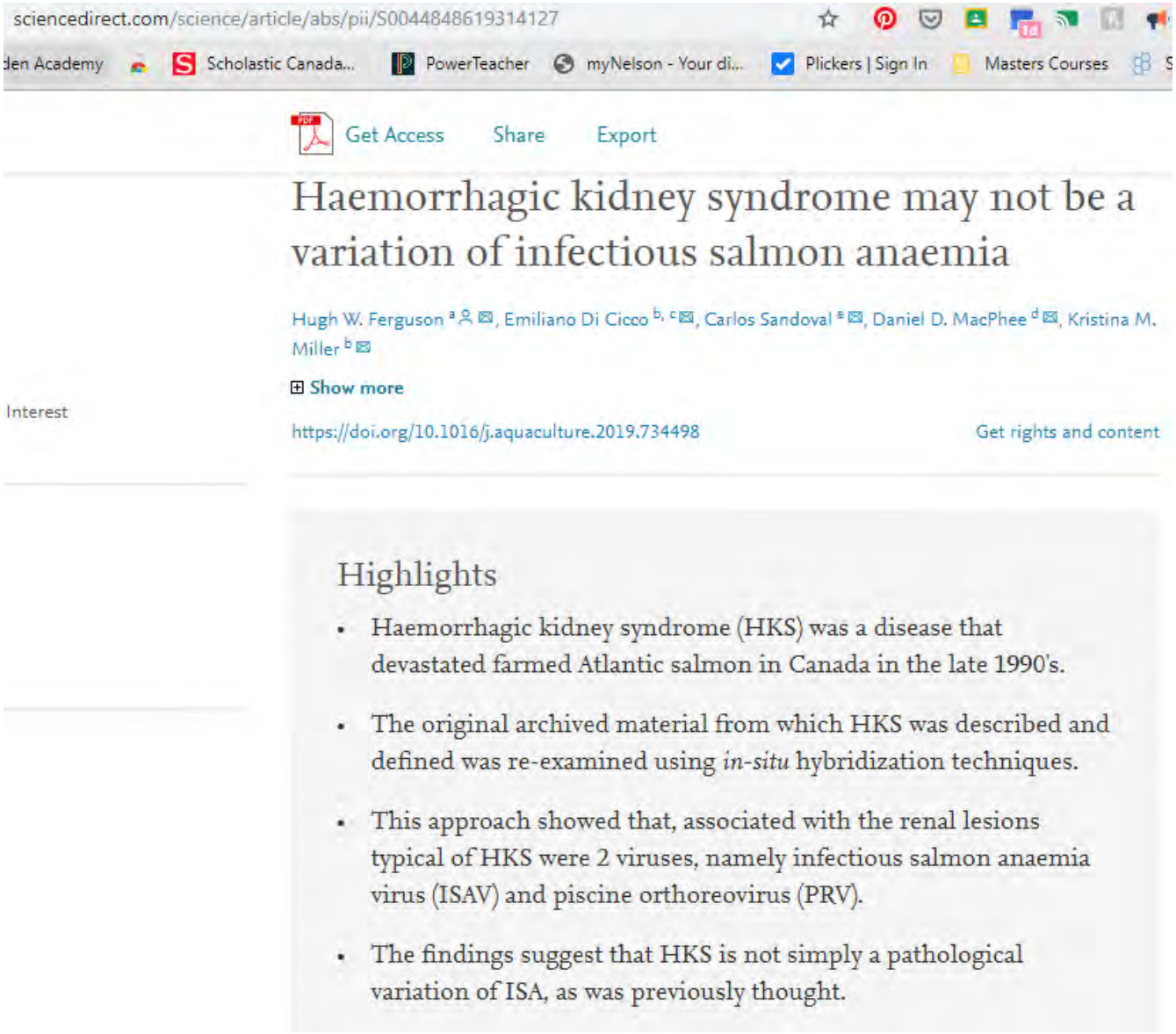
1.

From: http://www.ices.dk/sites/pub/Publication%20Reports/Expert%20Group%20Report/SSGEPI/2016/01%20WGPDMO%20-%20Report%20of%20the%20Working%20Group%20on%20Pathology%20and%20Diseases%20of%20Marine%20Organisms.pdf

A similar outcome can be seen in the 2018 report whereby supposedly biosecure hatcheries and government inspection of importation failed to result in eggs or smolt free of OIE reportable viruses: https://www.researchgate.net/publication/326467969_Report_of_the_Working_Group_on_Pathology_and_Diseases_of_Marine_Organisms_WGPDMO_13-17_February_2018_Riga_Latvia Of note is the admittance by the Canadian government that they allowed PRv imports that triggered mild disease in fish yet caused no significant financial losses to aquaculture companies. This is now a huge issue for Canada and BC in particular due to the recent finds by Dr Millers lab that shows that wild fish are seriously harmed by this mis-step. PRv is not screened in Maine, either. We do not known all the hosts of this virus. PRv in concert with ISAv, was only weeks ago, discovered to also cause Hemorrhagic Kidney Syndrome (Ferguson et al 2019)

Viruses from Egg Shipments:

Hatcheries are regularly caught harbouring, amplifying and even mutating fish viruses. The same is true for other pathogens. The industry has a long history of importing new non-endemic viruses to new regions. Allowing non-native salmon into BC Canada has resulted in introductions of EU strains of ISAv, PRv, SAv, (Kibenge 2016) and others we are only now discovering (Mordecai et al 2019). The same was true for Chile where the introduction of ISAv collapsed the entire salmon farming industry (~$2B USD) (Vike et al 2006). It seems little has changed, as more recently, in 2019, Washington State caught Cooke Aquaculture importing eggs from the same supplier that proponent wishes to use, that were infected with an exotic non-endemic EU strain of PRv This is also a huge issue in BC where screening of some strains of PRv infected fish were only just initiated. It took 3 court challenges to force regulatory agencies to do this. More than 80% of all aquaculture salmon in BC have this virus (Morton Unpubl Data). It is also in the broodstock of many major companies. It is not screened for in



Maine. Recently, the implications of this mis-step became apparent when it was discovered that this virus harms other marine fish severely, specifically Pacific salmon. It has now become an “electric third rail” for the industry and regulators. See Ferguson et al 2018 for a review. Dr Miller's lab in BC is expected to reveal more than the 3 harmful viruses she just published in 2019 (Mordecai et al 2019) that are new to science. While some of these new viruses she is working on may not cause mass mortality in Atlantic salmon they will likely be infectious to other fish with unknown outcomes.

The Fish are Clean Enough Syndrome :
The goal of RAS finfish aquaculture companies is to produce profit through the generation of fish in an artificial setting. Consumers then believe that these RAS fish are the same or similar to their wild counterpart. Nothing could be further from the truth. A wild fish has had to survive in a hostile environment, full of pathogens, predators, extreme weather, migrate past obstacles, etc and breed. A tanked fish simply has to survive in an environment where even a very sick fish can thrive and grow. Even the breeding has been removed from their requirements. A very sick tanked fish, loaded in viruses, unable to jump a waterfall in nature, or escape a predator can still be sold in the USA market.

Conversely, a very sick wild fish is a dead and/or eaten fish and/or unable to migrate and breed. Mixing the two results in what industry calls “spill over” and “spill back”. A pathogen that is at a super low frequency in the wild population, and often can not be found during survey work, due to the “struggle for life” quickly amplifies and/or mutates in a “bioreactor” such as a RAS system. The entire USA regulatory and monitoring system is designed to focus testing on “reportable” diseases that could affect trade. These 5 OIE viruses are but a tiny portion of the non-salmon and salmon pathogens that can and will be amplified in any RAS setting. Similarly, regulatory agencies and operators alike have very short lists of non-viral pathogens in their effective monitoring programs, all others are ad hoc and must result in serious mortality or pathology issues before being noticed. Many are missed.

The goal is to get fish to market, not make healthy fish that will not impact wild fish. The following is from a review of the Maine salmon breeding program:
“ The use of groundwater, reuse culture technologies, and effective biosecurity protocols has resulted in fish health certification for the facility and fish stocks. No mortality events or pathogens of regulatory concern have been reported on any fish health checks. All fish stocks were screened biannually for five viruses (IPNV, IHNV, ISAV, OMV, VHSV), along with Aeromonas salmonicida, Yersinia ruckeri, Renibacterium salmoninarum, Myxobolus cerebralis, and Ceratomyxa shasta. “ from : Wolter et al 2009

The proponent used this as a shining example of what is being done “right”. It is an example of where the bar is set, even for the Maine State salmon breeding program : mass mortality and population collapsing disease outbreaks. Anything above that is a success. Causes of regular mortalities and disease are not worth mentioning. Pathogens not important to financial considerations in an RAS system are basically ignored and flushed. Ironically, when one of the fathers of RAS development in the USA, Dr Steve Summerfelt (4th author on the above paper), finally did leave applied research in an academic setting and join the corporate world, he did so by joining a closed loop system called aquaponics and not any of the larger, semi-closed, or flow through systems being developed in the USA today. He chose what many believe is the best design and not what the proponent is offering to build.

The proponent then suggests that: “There have been no documented negative effects on wild fish stocks from the outflow pipe of these RAS facilities.” What he didn't offer was that virtually no monitoring or studies have been conducted for this facility or any other in North America or in fact the world.

Flow Through Design Issues :
All flow through systems that have ever been tried for rearing finfish suffer from a lack of negating this spill over and spill back effect. They invariable fail financially and/or cause alarming outbreaks of pathogens that end up “ineffectively sterilized” in the effluent. Even more alarming is that the intake and effluent pipes are in close proximity to one another. The UN calls the results of this concept “One Health”. If you have sick animals in husbandry you will have sick adjacent wild animals and these will then reinfect the animals in husbandry. The resulting “sick” ecosystem then impacts nearby and distant human health. Examples of the consequence of mixing wild animals and domesticated stocks abound. Disease flourishes due to predator exclusion, breeding non-requirements, less than perfectly effective drug treatments, etc in the unnatural husbandry environment. This then impacts the local wild animals. For example: Wild bison and cattle in Canada's midwest resulting in a Bovine TB epidemic spill-over and spill-back positive feedback loop that results in many endangered wood bison being culled annually and costs Canadian taxpayers millions. A parasite in Sweden was amplified in a hatchery and transferred to more than 40 rivers in Norway. These rivers then had to be Rotenon-ed (read, kill virtually every living thing in the river) and restocked. Even this did not kill every last one of the parasites, and these rivers despite having millions of dollars spent to recover them, have never recovered nor has the parasite been eradicated.

Pathogens in the Feed Pellets :

Annual Norwegian studies show that a significant percent (up to a 10% average) of the feed pellets produced and stored by industry carried human and fish pathogens (eg NIFES annual reports, EU Report 2003). Typically these were bacteria such as salmonella, or molds. Maine and the US Federal government has limited to no effective monitoring system for testing shipments for these pathogens.

Similarly, Chile banned all feed shipments from France (specifically, a major feed pellet plant owned by Sketting, a company the proponent suggested they might use) due to finding fish viruses that were still active and viable in the feed. These had originated from wild fish used to create the pellets.

Shark and Predator Attraction :

This, again, will be unmonitored and be an obvious issue when dumping 7 million gallons of chum a day into a bay where wild salmon will not be protected in tanks. Sharks attracted will be eating local wild fish. Many “salmon” aka porbeagle sharks migrate past the project location. This is a huge issue where I live in NL Canada.

Why So Big? Why Not Reduce the Size of the Project and Just Use Aquifer Water and Reduce the Pathogen Exposure Risks ? :

So, if the risk of pathogen introduction can be significantly reduced by simply using aquifer water, why not simply only use aquifer water as almost all of the recent RAS systems do? Well, it seems that the aquifer chosen was simply not able to supply enough water to satisfy the profit needs of the promoters. So, they decided to risk using seawater and surface water, from of all places, a nearby wild salmonid watershed and the same bay their own effluent will be discharged into. History has shown us this never ever works, if by “works” we mean make profit and provide jobs in a stable fashion and have limited and negligible disease outbreaks and thus reduced: culls, waste, antibiotic usage, etc

Mr Dagan is the CEO of one of the largest land based salmon developers in the World: Aqua MoAF https://salmonbusiness.com/23-months-from-egg-to-harvest-this-salmon-has-grown-30-miles-from-the-sea/

In a recent interview with a major trade magazine (Salmonbusiness.com) “estimates a production cost of USD 3.4 per kilo of live fish.“But remember that there are scale advantages,” he said. How big? “Very big. For 5,000 tonnes it would be USD 2.8 per kilo, and for 10,000 tonnes it would be USD 2.4-2.5 per kilo,” he added.”

So the simple answer is, the majority of risks of disease amplification and mutation due to surface water use, must be, in the promoters mind, manageable and bearable with regard to getting fish to market. What may not have been factored in is the ramifications of the many pathogens that simply make fish sick without causing mass mortalities. Wild fish exposed to the effluent will not live in a predator free tank, have access to antibiotics, etc. Moreover, no one is monitoring the health of exposed wildlife or even testing the effluent for known pathogens. This alarming fact was recently exposed in Canada by the 2018 Spring Reports of the Commissioner of the Environment and Sustainable Development to the Parliament of CanadaReport 1—Salmon Farming found here: https://www.oag-bvg.gc.ca/internet/English/parl_cesd_201804_01_e_42992.html The auditors were “shocked” by the total lack of monitoring of adjacent wild fish health, unlimited use of antibiotics, unlimited use of pesticides”. This is also the case in Maine. The Auditor also suggested that the results of the audit showed the most number of gaps of any audit she had ever conducted. Her expressions of horror when presenting the results to the Federal government need to be seen to be appreciated. https://www.youtube.com/watch?v=f2JZev5oYZ4 Again, the exact same situation exists in Maine.

Biocide Use Issues

Flow through RAS design does not allow compliance with Maine State Environmental Protection Chapter 514 which states:

C. A permit for aquatic pesticide use will be issued only if the applicant provides adequate protection for non-target species.

Incomplete List :

Many chemicals have been ignored by the proponent. These include chemicals banned in the EU such as ethoxyquin which is a common feed additive not banned in the USA but shown to be both carcinogenic and mutagenic. It has traditionally been used in Maine by Cooke Aquaculture. It also has been shown to cross the human blood brain barrier (Bohne unpublished data personal communication 2017). The amounts of this in many feed formulations can be extreme eg 1.5% w/w. The 3 main compounds from the breakdown of this pesticide are all biologically active and persist in the environment. See review by Blaszczyk et al 2013 and Bernhardt 2018. I have also submitted screenshots showing the ban being covered in the popular media.

Amounts Used Incomplete :

Many of the biocides the applicant wants to use have no estimated amounts. Moreover, Maine law does not restrict their use. Examples include: antiparasitics and antimicrobials in general.

The amounts discharged can be quite substantial with serious ramifications.

For example, a Cooke Aquaculture hatchery in St.Albans produces about 3 million 100g to 120 g smolt a year from about 5 million eggs. To do this in 2017 (last year data is available as seen here: http://isdm.gc.ca/opendata/aar/Land-Based%20and%20Freshwater%20Data%202017%20v1.xlsx and cited as DFO Antibiotic Data 2017) it used 180,750,000 milligrams of Florfenicol, 1,290,000 milligrams of Imvixa, 1,040,000 milligrams of Oxyteracycline, and 1,431,720,000 milligrams of formalin (to treat the eggs and not a human use antibiotic). All this ended up in what was once a large pristine Bay and what is now undoubtedly a soupy of antibiotic resistant bacteria.

This is 330,000 kgs or 330mt that required *183 KILOS of antibiotics*. This is typical. It is of special note that this hatchery uses UV and drum filtration of *aquifer only water*...not surface water known to be far more prone to serious pathogen infestation and yet wanting to be used by the proponent.

In contrast, 33,000 mt is what Nordic Aquafarms wants to generate. *100 times more.* Thus, the amount of antibiotics would be 18300 kilos or 18,300,000,000 mg by equivalency using the recently build, high tech, aquifer water only using, Cooke hatchery as a measuring stick. Yes 18.3 billion milligrams. This will, in all likelihood, result in much much more antibiotics being dumped in the bay than all of Belfast currently uses per year. Yet not a single monitoring study has been required nor any legal limits on antibiotic usage put in place. Japan does not allow the use of antibiotics in aquaculture - at all. Antibiotic use in aquaculture is a serious issue and part of the One Health initiative of the UN (One Health, WHO, United Nations, 2015).

What effects and affects this will have on the local marine and wildlife (and humans) is completely unknown. We do know that high usage of antibiotics in the freshwater and marine ecosystems by salmon farming has had dramatic effects in terms of cascade effects and antimicrobial resistance Wang et al 2019. A quick scan of the available scientific literature shows many studies where both human workers and ecosystems are affected.

For example, the province of NL Canada produces about 22,000 mt of salmon per year, yet used 5.9 billion milligrams of antibiotics in 2017, this does not include the hatchery phase of production which further elevates these numbers. See 2017 Marine usage rates in citations as an XL spreadsheet. This is 4 times what the same provinces used during the same year for terrestrial animal production (cows, sheep, pigs, goats, chickens, turkeys, ducks, etc) combined. Despite that in-feed administration is the primary method used during outbreaks, sick fish don't eat and many of these antimicrobials will end up in the feed waste and waste water. Many antimicrobials will not be administer to individual animals that are sick but instead the entire stock in the tank will be treated. This is called Broadcast treatments by some people and has been discouraged by the OIE, WHO, and UN in general. It has also been banned in many countries and the EU for all animals except fish. The reason is obvious. A vet can handle a sick pig, for example, but can't find, let alone treat a single sick fish in a giant tank. So, the entire tank is heavily dosed as a standard practice for many fungal and bacterial infections.

Lack of Monitoring for Antimicrobial Resistance :

Neither Maine nor the Federal USA government has an antimicrobial resistance monitoring program that would monitor the impacts of this project. Nor has the proponent offer any such monitoring program. These are obvious serious issues when one is considering the effects of billions of milligram being dumped in a small area annually thereby exposing *all* the local and migratory wildlife and marine life to such an effluent stream. Many studies have shown not only antimicrobial resistance jumping from freshwater fish tanks to human pathogens in a RAS aquaculture setting, even as close as NS Canada (McIntosh et al 2008), and marine based costal RAS (, Wang et al 2019), but serious impacts on antimicrobial resistance in the marine and freshwater environments (Paoni 2000, Rhodes et al 2008, Saavedra et al 2017, Buschmann et al 2012, Vincent et al 2014, Huang et al 2015, Henriques et al 2016, Bohle et al 2016, VKM Report 63 2016,). Please see Irish, Chilean, Norwegian studies eg Buschmann et al 2012, review by Pridgeon and Klesius 2012, Grant et al 2014. Human pathogens are already being discharged into the bay via the effluent, adding billions of milligrams of these antibiotics will have unknown as well as alarming predictable negative impacts (Gov Canada, The Interdepartmental Antimicrobial Resistance Policy and Science Committees 2002, Chee-Sanford 2009, Heuer et al 2009, Miranda et al 2013, review by Cabello 2013, Ivanova 2015, Done and Halden 2015). Monitoring of these impacts by government or the proponent in the Bay is unlikely as a monitoring framework does not exist within government despite repeated attempt to develop one (Smith 2008, Grant et al 2014), despite growing use and concern (WHO One Health 2012, Van Boeckle et al 2015).

Norway has restricted use to such an extent that 0.1 g/mt is the average antibiotic usage in 2018, while Japan has banned the use of antibiotics in aquaculture altogether. Maine has no legal limits on use and as a result, likely uses >50g/mt and as high as 280g/mt as is seen in Atlantic Canada. No legal limits will be imposed on the proponent. Use of 10s of billions of milligrams per year to produce 33,000mt should be expected based on nearby hatcheries in NB/NS/NL as reported by the DFO in Canada (see 2017 usage rates in DFO XL spreadsheet).

In addition to the effluent, spoiled medicated feed dumped, etc, as the fish move in the food distribution

chain, so do the antimicrobial resistant plasmids (Nespolo et al 2012).

Persistent in the environment and passes through the fish still active :

In addition, to simply not being ingested by sick fish that refuse to eat, or otherwise taken up by the fish, many antimicrobials will pass through the fish unaffected and have an extreme half life in cold water. More than 50% of many antimicrobials ingested will simply pass through the fish and be added to the effluent.

Chemicals for the Fish Farm

Note: Annual usage estimates represent approximate quantity required given a product is the only one used for this application. The quantities needed will be dependent on the site-specific conditions experienced which are difficult to establish prior to operations and are indicated as estimates only. Likely a fraction of the estimated annual use of each of these products will be used. All products listed will be used according to label.

Cleaners Detergents

Aqualife® Multipurpose Cleaner. A biodegradable, nonhazardous cleaner that is designed specifically for use in fish hatcheries, aquaculture facilities, fish & food processing plants, & agricultural farms. Active ingredients: sodium hydroxide (1-5%), the product is phosphate free, contains no volatile organic compounds and is NSF certified for use in food processing facilities. Used according to the label at dilutions of 1:20. Approximate annual use: 2232 gallons/year (8449 l/year).

Gil Save®. High-foaming chlorinated, alkaline, liquid detergent, Gil Save is designed for foam and high pressure spray cleaning of meat and poultry plants, breweries, dairies and canneries. It is a complete product containing alkalis, water conditioners, chlorine and high-foaming wetting agents. Gil Save is an effective cleaner of food processing equipment by removing fatty and protein soils, pectin, mold, yeast and organic greases. Active ingredients: sodium hydroxide (7- 9%), sodium hypochlorite (3-4%). Use according to label at concentrations of 0.2-3% (1⁄4-4 oz/gal). Approximate annual use: 678 gallons/year (2567 l/year).

Clean in Place (CIP)

Gil Super CIP®. A heavy-duty, chelated-liquid caustic cleaner for use in CIP, boil-out, soak, spray clean and atomization cleaning systems, Gil Super CIP is formulated to remove protein, fatty and carbonized soils typically found in dairy and food processing. Active ingredients: sodium hydroxide (49%). Used according to label at 0.1-3% (1/8-4 oz/gal). Approx. annual use: 5840 gallons/year (22107 l/year).

Gil Hydrox®. A concentrated organic, liquid acid cleaner, Gil Hydrox rapidly removes milk/beer stone, alkaline/hard water film and stains/protein build-up from dairy and food processing equipment. It is specially formulated for use in CIP, spray and acid rinse operations. Active ingredients: glycolic acid (29-31%). Used according to label at 0.3-1.5% (1⁄2-2 oz/gal). Approx. annual use: 5840 gallons/year (22107 l/year).

Disinfectants/Sanitizers

Bleach. Active ingredient: sodium hypochlorite (8%) in concentrated form. Typically used at 100-1000 ppm for general cleaning/disinfection. Approximate annual use: 1500 gallons/year (5700 l/year).

Ozone. Ozone can be dissolved into water to provide an aqueous ozone solution that is stable, safe,

easy to control, leaves no residue and has been granted GRAS approval by both the USDA and FDA for direct contact with food. This water containing ozone can replace chlorine as an antimicrobial agent or be used to supplement existing water rinses and achieve improved antimicrobial intervention. This is now a common application to sanitize fillet machines, cutting tables, knives, and all equipment that may be used in the seafood processing areas. Approximate annual use: TBD. Concentration in discharge = 0 ppm

Virkon® Aquatic. A powerful cleaning and disinfecting solution with efficacy against fish viruses, bacteria, fungi, and molds. Virkon® Aquatic is EPA registered (except in California where registration is pending) for the disinfection of environmental surfaces associated with aquaculture. Active ingredient: Potassium monopersulfate (21.4%). Used in accordance with label as a general cleaner and in footbaths. Working solution strengths normally range from 0.5% - 2.0%. Approx. annual use: 1100 lbs/year (500 kg/year).

Zep FS Formula 12167® Chlorinated Disinfectant and Germicide. A liquid chlorine sanitizer and deodorant for use in all types of food-handling establishments. Authorized as no rinse sanitizer for equipment. Provides deodorizing activity by destroying bacteria which generate many disagreeable odors. Can also be used to sanitize commercial laundry. Active ingredients: Sodium hypochlorite (5-10%) and sodium hydroxide (1-3%). Used according to label, effective at concentrations as low as 0.3% (1 oz/ 2 gallons). USDA applicable and EPA and Maine registered. Approx. annual use: 1980 gallons/year (7495 l/year).

Therapeutants

Compounds Potentially Used:

Note: the quantities needed will be dependent on the site-specific conditions experienced which are difficult to establish prior to operations and so are indicated as estimates only. All products listed will be used according to label use or a licensed veterinarian’s prescription.

Parasite-S, Formalin-F, and Formacide-B. (Formalin). Active ingredient 37% formaldehyde. Used periodically according to the label if needed to alleviate fish health issues due to saprolegniasis, external protozoa and monogenetic trematodes. Typical dose rates from 25 ppm to 1,000 ppm. Approximate annual use: 925 gallons/year (3500 l/year).

Finquel® or Tricane-S. (Tricaine methanesulfonate). Used periodically in accordance with the label to reduce stress on the fish when handling small numbers for examination. Typical dose rates of 15-330 mg/L. Approximate annual use: 1.1 lbs/year (500 g/year).

Halamid® Aqua. (Chloramine-T). Active ingredients N-chloro, p-toluenesulfonamide and sodium salt trihydrate. Used periodically according to the label if needed to alleviate fish health issues due to bacterial gill disease. Typical dose range 12-20 ppm. Approximate annual use:
1100 lbs/year (500 kg/year).

Ovadine® (PVP Iodine). A buffered 1% Iodine solution (Iodophor) specifically formulated for use in disinfecting fish eggs. It contains a 10% Povidone-Iodine (PVP Iodine) complex, which provides 1% available iodine. Used according to the label at dose rates of 50 -100 ppm as available iodine solution. Estimated usage: 160 gallons/year (600 l/year).

Compounds Rarely Used Only in Emergency Situations:

Praziquantel. Considered as 100% active. Can be used if fish are suffering from trematode/cestode infections. Typical dose ranges from 5-200 ppm depending on length of standing bath treatment. Used as needed/intermittent or emergency use only,
according to label use or as prescribed by a licensed veterinarian. Approx. annual use: 0 lbs/year (0 kg/year).

Potassium permanganate. Considered as 97% active. Can be used if fish are suffering from certain parasites and fungal infections in younger fish life-stages. Typical dose range 1.5-2.5 ppm. Used as needed/intermittent or emergency use only, according to label
use or as prescribed by a licensed veterinarian. Approx. annual use: 0 lbs/year (0 kg/year).

Terramycin® 200. (oxytetracycline dehydrate, 44% active): Can be used as an in-feed treatment (maximum of 0.08 g active oxytetracycline/kg fish/day) if fish are suffering from certain bacterial infections. Used as needed/intermittent or emergency use only, according to label use or as prescribed by a licensed veterinarian. Approx. annual use: 0 lbs/year (0 kg/year).

Aquaflor®. (florfenicol; 50% active). Can be used as an in-feed treatment (maximum of 15 mg/kg fish/day) if fish are suffering from certain bacterial infections. Used as needed/intermittent or emergency use only, according to label use or as prescribed by a licensed veterinarian. Approx. annual use: 0 lbs/year (0 kg/year).

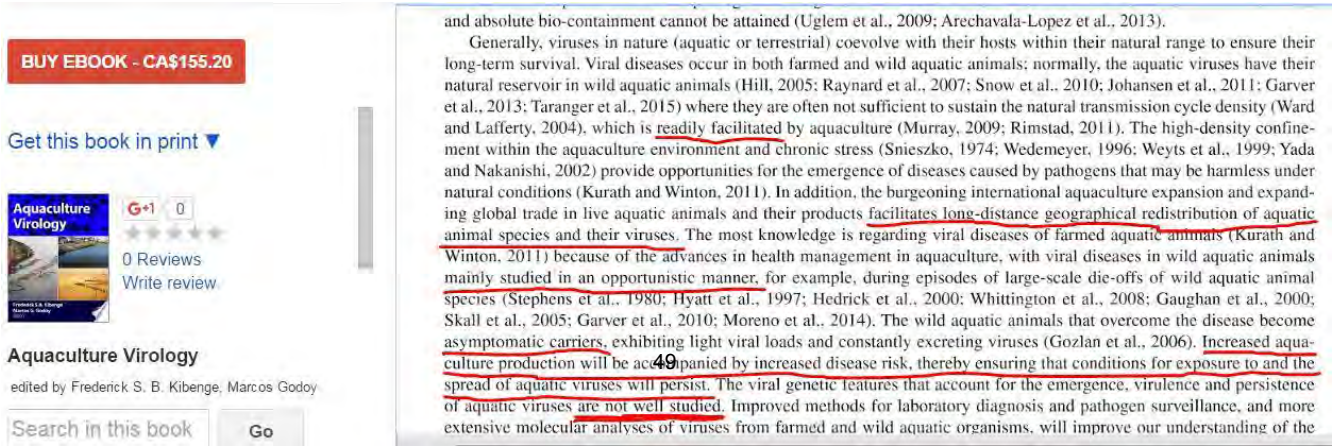
Romet® 30/Romet® TC. (sulfadimethoxine/ormetoprim, 30% active or 20% active, respectively). Can be used as an in-feed treatment (maximum of 50 mg/kg fish/day) if fish are suffering from certain bacterial infections. Used as needed/intermittent or emergency use only, according to label use or as prescribed by a licensed veterinarian. Approx. annual use: 0 lbs/year (0 kg/year).

WasteWater Treatment

Formic Acid (85%). Used for pH correction of fish processing water prior to disinfection with sodium hypochlorite. Approx. annual use: 18200 gallons/year (69000 l/year).

Bleach. Active ingredient: sodium hypochlorite (15%). Used to disinfect water used in fish processing. Applied at a concentration of 50 mg/l. Estimated discharge concentration: 0.4 mg/l. Approx. annual use: 14800 gallons/year (56000 l/year).

MicroC® 2000. (1.1 million mg/l COD). A non-hazardous, green chemical developed specifically for use as an electron donor / carbon source for wastewater denitrification applications. It is used as a supplemental carbon source in wastewater treatment plants to stimulate denitrification processes. Approx. annual use: 1.0 million gallons/year (3.8 million l/year).



2.1.2. Survival outside the host ISAV has been detected by reverse-transcription polymerase chain reaction (RT-PCR) in seawater sampled at farming sites with ISAV-positive Atlantic salmon (Kibenge et al., 2004). It is difficult to estimate exactly how long the virus may remain infectious in the natural environment because of a number of factors, such as the presence of particles or substances that may bind or inactivate the virus. Exposing cell culture-propagated ISAV to 15°C for 10 days or to 4°C for 14 days had no effect on virus infectivity (Falk et al., 1997). 2.1.3. Stability of the agent (effective inactivation methods) ISAV is sensitive to UV irradiation (UVC) and ozone. A 3-log reduction in infectivity in sterile freshwater and seawater was obtained with a UVC dose of approximately 35 Jm–2 and 50 Jm–2, respectively, while the corresponding value for ISAV in wastewater from a fish-processing plant was approximately 72 Jm–2. Ozonated seawater (4 minutes with 8 mg ml–1, 600–750 mV redox potential) may inactivate ISAV completely. Incubation of tissue homogenate from diseased fish at pH 4 or pH 12 for 24 hours inactivated ISAV. Incubation in the presence of chlorine (100 mg ml–1) for 15 minutes also inactivated virus (Rimstad et al., 2011). Cell culture-isolated ISAV may survive for weeks at low temperatures, but virus infectivity is lost within 30 minutes of exposure at 56°C (Falk et al., 1997).

https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_isav.pdf


69.17° W
44.48° N
44.32° N
68.85° W
Morrill
Warren Brook
Passagassawakeag River
137
Belfast
Belfast Bay
Belfast Municipal Airport
Little River
Piper Stream
Tilden Pond
LEVENSELLER MOUNTAIN
673 ft
Searsport
Long Cove
Stockton Harbor
Sears Island
Islesboro Island
N
0
2.6 km (1.6 miles)
DATA BASIN
MAINE
Augusta
Legend
Important Anadromous Fish Habitat
Displaying: IncDesc
Multiple Species (Non-Alosid)
Single Species (Non-Alosid)
Top 5% Alosid
Top 5% Alosid plus Non-Alosid Species
Atlantic Salmon Habitat Suitability
Proportion of Habitat > 10%

NOTE - Th s map contains two datasets: “Important Anadromous Fish Habitat, Northeast U.S.” and “Atlantic Salmon Rearing Areas, Maine”

These dataset are part of a suite of products from the Nature’s Network project (naturesnetwork.org). Nature’s Network is a collaborative effort to identify shared priorities for conservation in the Northeast, considering the value of fish and wildlife species and the natural areas they inhabit. “Important Anadromous Fish Habitat” and “Atlantic Salmon Rearing Areas, Maine” are two input used in developing “Lotic Core Areas, Stratifi d by Watershed, Northeast U.S.” that is also part of Nature’s Network. Lotic core areas represent intact, well-connected rivers and stream reaches in the Northeast and Mid-Atlantic region that, if protected as part of stream networks and watersheds, will continue to support a broad diversity of aquatic species and the ecosystems on which they depend. The combination of lotic core areas, lentic (lake and pond) core areas, and aquatic buffers constitute the “aquatic core networks” of Nature’s Network. These and other datasets that augment or complement aquatic core networks are available in the Nature’s Network gallery: https://nalcc.databasin.org/galleries/8f4dfe780c444634a45ee4acc930a055.

Important Anadromous Fish Habitat:

Intended Uses

Th s dataset is primarily intended to be used in conjunction with the Nature’s Network product “Lotic Core Areas, Stratifi d by Watershed, Northeast U.S.” to better understand the importance of core areas to anadromous fish. It also can be used on its own, or in conjunction with the Atlantic salmon dataset, to identify priority watersheds for anadromous fish.

Description and Derivation

Th s dataset is a combination of the following two products:

1) Habitat for Atlantic sturgeon, short-nosed sturgeon, and sea-run (salter) brook trout.

Atlantic and shortnose sturgeon are federally listed endangered species. Sea-run or salter brook trout, which undertakes seasonal migrations from the ocean into rivers and streams, is also of conservation concern. Occurrence was compiled by Dauwalter et al. (Dauwalter, Daniel C., Carolyn J. Hall, Jack E. Williams. 2012. Assessment of Atlantic Coast watersheds for river herring and diadromous fish conservation. Trout Unlimited fi al report to National Fish and Wildlife Foundation. Trout Unlimited, Arlington, Virginia.)

2) Top 5% of watersheds for alewife, blueback herring, and American shad (collectively referred to as “alosids”).

Th s component of the product consists of streams and rivers in the top 5% of watersheds for conservation action for river herring (alewife and blueback herring) and Azmerican shad, based on a prioritization developed by The Nature Conservancy (TNC). The 2015 TNC analysis is intended to identify areas of high anadromous fish conservation potential along the Atlantic Coast. For the analysis, a suite of metrics was calculated in each subwatershed (USGS 12-digit hydrologic units or “HUC12s”) of the U.S. Atlantic Coast to measure population and habitat factors which are relevant to these fish. The high priority subwatersheds are areas where conservation activities to support these fish could have the greatest impact. They are intended as a regional-scale screening tool to be used in concert with local-scale information and expertise; they are not a prescription for any particular management action.

The metrics used in the alosid analysis fall under four categories:

1) Population – recent run count or occurrence, or historical occurrence (as compiled by Dauwalter et al. 2012)

2) Habitat Quantity and Access – e.g., wetland extent, percent of stream reaches in the subwatershed with unrestricted downstream access (no barriers) to the ocean

3) Water Quality – extent of impervious surface

4) Water Quantity – potential dam impacts on stream and river fl w based on total upstream dam storage capacity

These factors were then weighted by importance for each species based on expert knowledge. The results of the simple weighted ranking prioritization algorithm were then binned into 5% tiers for each species; the top tier is considered to have the greatest restoration potential. The top tiers for each of the three species were also combined to result in a combined Top 5% representing the highest tier for one or more of the three species. The metrics and results can be viewed on the Fish Habitat Decision Support Tool, http://www.fishhabitattool.org/, in the Atlantic Coastal Fish Habitat Partnership section.

Atlantic Salmon Rearing Areas:

Intended Uses

This dataset is primarily intended to be used in conjunction with the Nature's Network product "Lotic Core Areas, Stratified by Watershed, Northeast U.S." to better understand the importance of core areas to Atlantic salmon. It also can be used on its own, or in conjunction with the dataset "Important Anadromous Fish Habitat, Northeast U.S." to identify priority watersheds for anadromous fish.

Description and Derivation

The dataset consists of stream reaches that are predicted to contain greater than 10% Atlantic salmon rearing habitat, based on a GIS-based habitat model developed by the U.S. Fish and Wildlife Service and the National Oceanic and Atmospheric Administration (NOAA). The model assesses salmon rearing habitat throughout the range of the Gulf of Maine Distinct Population Segment (DPS) of Atlantic salmon, which is federally listed as an endangered species. The model was developed using data from habitat surveys conducted in the Machias, Sheepscot, Dennys, Sandy, Piscataquis, Mattawmkeag, and Soudabscook Rivers. The model uses reach slope derived from contour and digital elevation model (DEM) datasets, cumulative drainage area, and physiographic province to predict the total amount of rearing habitat within a reach. The variables included in the model explain 73% of the variation in rearing habitat. More details about the model are available at: https://www.greateratlantic.fisheries.noaa.gov/prot_res/altsalmon/Appendix%20C%20-%20GIS%20Salmon%20Habitat%20Model.pdf.

All citations are in these two folders:
https://drive.google.com/open?id=16IRTPpv2wG8wHoZmyaQMX8-aAKZIQXdi

and

https://drive.google.com/open?id=1-I6sR5y-Mzi3RlCk6TQFpE5rG3pHMxa_

13 Forest Rd
Lumsden, NL A0G-3E0 Canada
(709) 530-2637
Newfoundlander_1@Hotmail.com

William H. Bryden

SKILLS

Up-valuing existing factories through microbial production, mycoremediation, species cultivation development, ecoponics development and design, customized class 10 biosecure lab design and construction, office/scientific/lab software, culturebank development and maintenance, business plan development, photography, aviation, permaculture land development.

EXPERIENCE

Atlantic Mushrooms Inc, Lumsden, NL — *Director Research and Development*

April 2016 - PRESENT

- Developing new anexic cultures
- Designing and developing biosecure class 10 equivalent labs and microbial factories.
- Directing a team of microbial researchers
- Training lab staff
- Contamination source determination
- Genetic analysis of strains
- Exploring new microbial cultivation techniques
- Liaison with provincial production and research facilities

Consultant, Lumsden, NL

2012 - Present

- Risk assessment for aquaculture facilities.
- Environmental Assessment Review.
- Surveillance and monitoring of aquaculture facilities.

EDUCATION

Memorial University, St. John's, NL — *B.Sc Ecology and Evolutionary Biology*

1991 - 1996, St. John's

Honors program. 27 courses at the undergraduate and cross referenced graduate level focused on ecology, parasitology, molecular genetics, physiology

Honors research funded by provincial government, presented at National Zoology Conference.

I certify under penalty of law that this document and all attachments were prepared under my direction or supervision in accordance with a system designed to assure that qualified personnel properly gather and evaluate the information submitted. Based on my inquiry of the person or persons who manage the system, or those persons directly responsible for gathering the information, the information submitted is, to the best of my knowledge and belief, true, accurate, and complete. I am aware that the re are significant penalties for submitting false information, including the possibility of fine and imprisonment for knowing violations.

Bryden

Date: Dec 13 2019

Printed Name: Bill Bryden

Title:

Parties Assisting:

Name: Address: Signature:_____

Name: Address: Signature:_____

da Post / Po
WESLEY
281
WESLEYV
GST/TPS

2019/12/11 12:09.
CC/CC5622 W/G

N 1@$39.34
Priority Worldwide

Actual Weight 0.062kg
To 04915

This is your Tracking #
3 0 5 2 7 5 2 0 7 5 3 0

Coverage declined

Expected Delivery: 2019/12/13 by 16:30

N 1@$3.25 $3.25
Fuel Surcharge

For complete terms and conditions consult the Canada Postal Guide at www.canadapost.ca or any Post Office.

Sender warrants that the shipped item(s) do(es) not contain non-mailable matter.

SUBTL $42.59
TOTAL TAX $0.00
TOTAL $42.59

orité

caractères d'imprimerie. Appuyer fermement SVP.

ement No.
te SCP

SUITE/APP/BUREAU

Postal Code A0G 3E0
Code postal

530 2637

P/BUREAU

ZIP/Postal Code 04915
Code postal

491 6839

International delivery by FedEx Express
Livraison internationale par FedEx Express

Fe

4 Shipment Information / Informations sur l'envoi

Please describe the contents of your shipment in the box below. If the description of the contents is too vague, r language other than English, customs authorities may hold the shipment for further inspection. / Veuillez décrire envoi dans la case ci-dessous. Si la description est trop vague, manquante ou dans une langue autre que l'angla douanières pourraient retenir l'envoi pour inspection plus détaillée.

Total Weight / Poids total 0.063 kg Dimensions 40 / 25

Itemized List of Contents / Liste détaillée du contenu	Country of Manufacture / Pays de fabrication	Declare Valeur déc
Document		$
		$
		$
		$

Total Declared Value for Carriage / Valeur déclarée pour le transport (CAD) $ 0

Total Declared Value for Customs / Valeur totale déclarée à la douane (CAD) $

WARNING: False representation on any customs document is considered an offence.
AVERTISSEMENT : Toute représentation frauduleuse d'un document de douane est une infraction.

5 Packaging / Emballage

☑ *Priority* Worldwide Envelope / Enveloppe *Prioritè* Mondial ☐ *Priority* Worldwide Pak / Pak *Prioritè* Mondial ☐ Other / Autre

e.g., box, envelope, tube, etc. / ex.,

6 Sender's Authorization / Autorisation de l'expéditeur

The sender agrees that the Terms and Conditions (on reverse) apply and that the carrier's liability is limited in accord Sender warrants no dangerous goods are being shipped. / L'expéditeur convient que les Conditions et Modalités stip s'appliquent et que la responsabilité du transporteur est limitée en vertu de ces dernières. L'expéditeur garantit en o ne contient aucune marchandise dangereuse.

Sender's Signature / Signature de l'expéditeur Bryden Date Dec

CPC Use Only / À l'usage de la SCP seulement	Date In / Date de réception	Time In / Heure de réception	
	2019/12/11	☑ AM ☑ PM	☑ Cash, Credit Card, Other / Comptant, carte de cré ☐ Bill Account / Facturer au compte ☐ Meter /

Cost Centre / Centre de coûts	Postage Rate / Tarif d'affranchissement	Declared Value for Carriage Surcharge / Supplément - valeur déclarée pour le transport	Other Surcharges / Autres suppléments	Total
005622	$	$ 0	$	$

Tracking Number / Numéro de suivi 3052 7520 7530

FedEx Form ID No. / N° du formulaire FedEx

DTU Vet
National Veterinary Institute

EXHIBIT A-8

Treatment of wastewater from fish slaugtherhouses

Evaluation and recommendations for hyginisation methods

Helle Frank Skall and Niels Jørgen Olesen

National Veterinary Institute, Technical Univeristy of Denmark

June 2011

Danmark og EU investerer i bæredygtigt fiskeri og akvakultur

Projektet er støttet af Fødevareministeriet og EU

Ministeriet for Fødevarer, Landbrug og Fiskeri

Den Europæiske Fiskerifond

Introduction

Prevention of fish diseases is essential for profitable operation of aquaculture facilities. Historically, pathogens (disease-causing bacteria, viruses, parasites or fungi) derived from fish processing companies have been involved in transferring infection. Not just the processing of Danish fish (mainly aquaculture fish) pose a significant risk, also increased globalization with escalating transport of fishery products enlarge the risk of transfer of pathogenic micoorganisms, which ae a risk not only for aquaculture fish but just as much to wild fish. In Denmark, especially infection with the highly loss-causing fish disease VHS has been crucial. Several disease outbreaks could be associated with release of infectious waste from cutting plants that had slaughtered/processed VHS infected fish. Based on this, the National Veterinary and Food Administration in 2005 introduced requirements for either the sanitisation or percolation of wastewater from fish processing plants. The claim was however introduced without that there was a final decision on whether the proposed methods were effective under Danish conditions and without assessing the business economics of the claim. The requirement would apply to existing companies from September 2008. It was, however, difficult to implement the required methods, as no one in the industry knew what methods were most successful based on financial, operational and disease prevention rationale.
The Danish Veterinary and Food Administration has per August 2008 amended the order requiring that all fish processing companies now base their wastewater treatment of on percolation. This is far from possible at many of the existing Danish fish processing companies because of location and soil conditions. Based on this the order opens the possibility to apply for dispensation to sanitize the wastewater instead. However, there are no pre-approved methods, and it is therefore imposed on the industry to generating knowledge in the field. All companies must by the end of 2010 meet the requirements.

Objectives of the project

- To provide knowledge about existing and new methods to sanitize wastewater from cutting plants.
- To evaluate the disinfecting effect of wastewater perculation under laboratory conditions.
- Through the above to achieve the best possible security measures to avoid spread of fish diseases to both aquaculture and wild fish.

Content of the project

- Desribe and evaluate the current methods for disinfection of wastewater from fish cutting plants.
- Recommend methods which are acceptable based on disease transfer risks.
- Assess the disinfecting effect of percolation under laboratory conditions.
- Produce a report, which describes the different methods and recommendations to future requirements taking into account the optimum effectivity, efficiency, reliability and economy.

This report deals with the objective described in the first bullet point and thus do not take solid waste into consideration. Perculation will be described in further details in a following report concerning the second bullet point.

Conclusions

Based on literature studies a number of methods for sanitazion of process wastewater from fish slaughterhouses/cutting plants are acceptable. Most of these methods are described in the historic Danish legislation "Bekendtgørelse nr. 755 af 28/07/2005 om bekæmpelse af visse smitsomme sygdomme hos fisk" (Ministerial orden no. 755 of 28/07/2005 regarding control of certain infectious diseases in fish) annex 1 and are approved according to the present Norwegian legislation "FOR 1997-02-20 nr 192: Forskrift om desinfeksjon av inntaksvann til og avløpsvann fra akvakulturrelatert virksomhet" (Regulation 1997-02-20 no 192: Regulation concerning disinfection of intake water and outlet water from aquaculture related enterprises). The methods are able to reduce the amount of virus 3 log for VHSV, IHNV, and ISAV. IPNV may not necessarily be reduced by 3 log using the recommended methods. For the pathogenic bacteria *Yersinia ruckeri*, *Aeromonas salmonicida* and *Vibrio anguillarum* the methods are also acceptable.

pH treatment:

a) Mechanic separation (≤ 300 µm filter) followed by acid treatment to pH ≤ 3.0 for ≥ 8 hours.

b) Mechanic separation (≤ 300 µm filter) followed by basic treatment to pH ≥ 12.0 for ≥ 24 hours.

Chlorination:

a) Mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 10 mg/l residual chlorine after 15 minutes treatment.

b) Mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 2 mg/l residual chlorine after 25 minutes treatment.

Heat treatment:

a) 65°C for 10 minutes.
b) 70°C for 5 minutes.
c) 75°C for 4 minutes.
d) 80°C for 3 minutes.
e) 85°C for 2 minutes.
f) 90°C for 1 minute.
g) 95°C for 45 seconds.
h) 100°C for 30 seconds.

N.B. Proper stirring is necessary to make certain that no pockets with inappropriate heating exist.

UV-irradiation:

For wastewater treatment the method cannot at present be recommended as sanitizing method, as wastewater will be too organic polluted without a significant clarification before irradiation.

Ozone:

Mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by ozone treatment
a) fresh water: ≥ 0,15 mg/l residual ozone after 15 minutes treatment.
b) salt water: ≥ 0,2 mg/l TRO (total residual oxidants) after 15 minutes treatment.

Percolation	Although generally considered a safe method for wastewater sanitation it has not been possible to find any references describing the decimating effect of percolation on fish pathogenic viruses. As a substitute for IPNV, it has not been possible to find publications describing the effect of percolation on other birnaviruses. The effect of percolation on other viruses has not been looked into. It is therefore not possible in this report to validate if the procedure is safe to use.

Legislation

Danish legislation

In the historic legislation "Bekendtgørelse nr. 755 af 28/07/2005 om bekæmpelse af visse smitsomme sygdomme hos fisk" (Ministerial orden no. 755 of 28/07/2005 regarding control of certain infectious diseases in fish) annex 1 describes different disinfection methods that, at that time, were allowed to use. These are:

Formic acid (HCOOH):	Mechanic separation (≤ 300 µm filter) followed by treatment using formic acid to a) pH ≤ 4.0 for ≥ 24 hours, or b) pH ≤ 3.5 in ≥ 8 hours.
NaOH:	Mechanic separation (≤ 300 µm filter) followed by treatment using NaOH to pH ≥ 12.0 for ≥ 24 hours.
UV-irradiation:	a) chemical precipitation (Fe- and/or Al-salts) followed by UV irradiation of the supernatant using an UV-dose ≥ 25 mWs/cm^2. b) Mechanic separation (≤ 40 µm filter) followed by UV irradiation of the supernatant using an UV-dose ≥ 25 mWs/cm^2.
Chlorination:	a) mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 10 mg/l residual chlorine after 15 minutes treatment. b) mechanic separation (≤ 300 µl filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 2 mg/l residual chlorine after 25 minutes treatment.
Heat treatment:	a) 65°C for 10 minutes. b) 70°C for 5 minutes. c) 75°C for 4 minutes. d) 80°C for 3 minutes. e) 85°C for 2 minutes. f) 90°C for 1 minute. g) 95°C for 45 seconds. h) 100°C for 30 seconds.

Percolation

In the present legislation "Bekendtgørelse nr. 755 af 08/07/2008 om autorisation og drift af akvakulturbrug og –virksomheder" (Ministerial order no. 755 of 08/07/2008 regarding authorisation and operation of aquaculture farms and – enterprises) fish cutting plants are according to § 14 obliged to percolate process wastewater. Wastewater may, after permission from the Danish Veterinary and Food Administration, also be discharged to seawater. Dispensation from percolation of wastewater can, according to § 15, be permitted if the wastewater is disinfected and the chosen method result in a complete inactivation of infectious matters.

Legislation in the USA

Dr. P. Gary Egrie from the USDA APHIS Veterinary Services, informed that the Environmental Protection Agency (EPA) has regulatory authority for effluents into public waterways, however they have not developed regulations or recommended methods to address the discharge of aquatic animal pathogens from fish slaughterhouses.

Legislation in Norway

In Norway the legislation "FOR 1997-02-20 nr 192: Forskrift om desinfeksjon av inntaksvann til og avløpsvann fra akvakulturrelatert virksomhet" (Regulation 1997-02-20 no 192: Regulation concerning disinfection of intake water and outlet water from aquaculture related enterprises) regulates the effluents from fish slaugtherhouses. In this legislation it is described in § 9 that outlet water from fish slaugtherhouses/fish cutting plants has to be filtered through a grating before further treatment. The size of the grating has be ≤ 1 mm. In § 10 hourshe demands for the methods for desinfection of the outlet water after filtration are described. The metods have to be documented through scientific documentation based on relevant experimental test designs (water quality, Temperature etc.) to induce at least 3 log (99,9%) inactivation of *Aeromonas salmonicida* subsp. *salmonicida* and ISAV, or based on a dose-repons curve for IPNV it is likely that ISAV is inactivated likewise. In § 11 on demands to the technical equipment it is described that approved technical equipment shall at least be equipped with security measures which guaranties that the disinfectant's/method's "concentration" (mg/l, mWs/cm2, °C etc.) and time are kept. Furthermore a safety device against malfunction and a recording unit has to be installed.
According to § 8 the National Veterinary Institute (Veterinærinstituttet) is responsible for approval of methods. The approved methods can be found on the Norwegian Food Safety Authority (Mattilsynet) homepage and are as follows:

Formic acid (HCOOH):	a) pH ≤ 4.0 for ≥ 24 hours, or b) pH ≤ 3.5 in ≥ 8 hours.
NaOH:	≥ 12.0 for ≥ 24 hours.
UV-irradiation:	Chemical precipitation followed by UV irradiation of the supernatant using an UV-dose ≥ 25 mWs/cm^2.
Chlorination:	a) mechanic separation or chemical precipitation followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 10 mg/l residual chlorine after 15 minutes treatment. b) mechanic separation or chemical precipitation followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 2 mg/l residual chlorine after 25 minutes treatment.

Heat treatment:	a) 65°C for 10 minutes. b) 70°C for 5 minutes. c) 75°C for 4 minutes. d) 80°C for 3 minutes. e) 85°C for 2 minutes. f) 90°C for 1 minute. g) 95°C for 45 seconds. h) 100°C for 30 seconds.

These methods are basically the same as the methods in the former Danish legislation.

Ozone is not on the list for disinfection methods for wastewater from fish slaugherhourses or cutting plants. This method is approved for disinfection of wastewater from infection trial facilities handling A-, B- and C diseases, exotic and unknown pathogens. In facilities like this the following methods are approved:

Heat treatment:	d) 80°C for 4 minutes. e) 85°C for 3 minutes. f) 90°C for 2 minute. g) 95°C for 1 minut. h) 100°C for 30 seconds.
Chlorination:	a) freshwater: ≥ 25 mg/l residual chlorine after 30 minutes treatment. b) sea water: ≥ 35 mg/l residual chlorine after 30 minutes treatment.
Ozonation:	a) freshwater: ≥ 0,15 mg/l residual ozone after 15 minutes treatment (corresponds to a C T value of 135 mg*s/l). b) sea water: ≥ 0,2 mg/l TRO (total residual oxidants) after 15 minutes treatment (corresponds to a C T value of 180 mg*s/l).

Slaugther offals, which are not to be used as feed/food, shall be treated in accordance with the provisions laid down in regulation on animal by-products. This also applies to the organic sludge produced in conjunction with treatment of wastewater.

Legislation in UK

In England the solid waste from fish slaugtherhouses/cutting plants is regulated by the Animal By-products regulations. The Animal By-Products legislation dictates how different categories of solid waste are disposed of, including tissues from diseased animals. The latter would be Category 2 waste, and waste from apparently healthy animals would be Category 3 waste.

Assuming a fish processing plant is processing category 3 animal by-product/material then all waste/wash water is controlled under waste/environmental legislation and the operator needs to contact the Environment Agency (EA) regarding controls on this. If it is processing category 2 animal by-product/ material then it needs to have a pre-treatment process - essentially a 6 mm mesh with all material caught in the drain trap disposed of as category 2. However again once the liquid has passed through the trap it is a matter for environmental regulation to control it.

The EA would expect that the wash water would go to a foul sewer under a Trade Effluent Agreement with the sewerage undertaker (local water company). The sewerage undertaking might require some filtering or

other treatment to be done before discharge to sewer. The wash water should not be sent to surface water drainage or be directly discharged to a river. However, if the premises were 'in the middle of no-where' with no sewerage links then the company would need a discharge consent from the Environment Agency; they would probably require some pre treatment dependent upon analysis of the proposed discharge. The alternative would be to tanker off site to a sewage works. There does not seem to be any requirements to treat the liquid on site regarding inactivation of pathogens (personal communication from Peter Dixon, CEFAS).

Materials and Methods

These procedures in the former Danish legislation will be evaluated, based on a literature review, together with potential other procedures for their capability to decimate fish pathogens. The underlying basis will be the non exotic fish pathogens listed in "Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals". These diseases are viral haemorrhagic septicaemia (VHS) caused by VHS virus, infectious haematopoietic necrosis (IHN) caused by IHN virus, koi herpes virus (KHV) disease caused by KHV and infectious salmon anaemia (ISA) caused by ISA virus.

For the Danish aquaculture industry there are a number of other important pathogens such as e.g. infectious pancreatic necrosis virus (IPNV) causing IPN, *Renibacterium salmoninarum* causing bacterial kidney disease, *Aeromonas salmonicida* causing furunculosis, *Yersinia ruckeri* causing enteric redmouth disease and *Flavobacterium psychrophilum* causing rainbow trout fry syndrome.

Some of these diseases are furthermore listed in the Danish "Bekendtgørelse nr. 975 af 13/08/2010 om lister over smitsomme sygdomme til lov om hold af dyr" (Ministerial order no 975 of 13/08/2010 concerning listing of infectious diseses in relation to legislation regarding keeping of animals).

Different kinds of methods for desinfection

Acid

Lowering of the pH to an unfavourable niveau for microorganisms can be done by using organic acids and inorganic acids. For the inorganic acid the effect is solely based on the pH denaturing the proteins. Beside the pH effect the organic acids will enter the fish cells more easily than inorganic acids and thereby enhance the speed of which autolysis of the cells occur, whereby e.g. viral particles within the cells will be reached.

An example of an often used organic acid is formic acid, HCOOH. Formic acid is the simplest carboxylic acid, named after the Latin word for ant, formica, as this is the acid produced by the ant. Formic acid has an acid dissociation constant at the logarithmic scale (pK_a) of 3,7 and is a week acid.

An example of an inorganic acid that often is used to lower pH is hydrochloric acid, HCl. Depending on the source the pK_a for HCl is stated as -7 to -3 which means that in water the HCl will be completely dissociated into H^+ and Cl^-.

If the wastewater is pretreated for example by filtration so lumps of waste (e.g. fish flesh) are not present, there will probably not be much difference in the effect whether an organic or inorganic acid is used to lower the pH.

Base

A high pH is unfavourable for microorganisms. To raise the pH sodium hydroxide, NaOH, can be used. NaOH is a strong base with a pK_a of approximately 13. NaOH is very soluble in water with liberation of heat. NaOH does not react with iron, but it will react with transition metals such as e.g. aluminium.

UV irradiation

UV light is divided into three ranges UV-A (320 – 400 nm), UV-B (280 – 320 nm) and UV-C (190 – 280 nm). The highest capacity to damage microorganisms is found in the UV-C band. The damaging effect of UV light is caused by the altering effect the UV-light has on nucleic acids. When the light is absorbed by the DNA/RNA molecule dimerization of two pyrimidine molecules can occur. This will lead to blocking of the replication (reviewed in 68).

Different kinds of microorganisms are more or less susceptible to UV light. Generally speaking, the suscpetibilty is higher for growing bacteria than for viruses and bacterial spores.

The intensity of the UV light and the time of irradiation are important factors for the ability of the light to inactivate the microorganism. The UV dose is the multiplication of the intensity and the time and is a measure for the amount of energy which reaches a surface. The dose is often expressed as mWs/cm^2.

As UV light is not killing the microorganism there is a possibility for the microorganism to repair the damages of the DNA by photoreactivation. Light in the visible spectrum is able to activate enzymes which can repair the damages induced by the UV light (reviewed in 68). Keeping bacteria in the dark after UV irradiation for 15 hours will inhibit the photoractivation (72). There are, though, also different processes that may occur in the dark in bacterias that are able to repair damages. There is as such a possibility for bacterias to regain the ability to multiply after being exposed to UV light. Increasing the UV dose will decrease the ability for the bacteria to repair itself (72).

Chlorination

Chlorine is a well known disinfectant used for decades both in the industry as well as in the household. The effectivity of chlorine is dependent on factors such as pH, temperature, suspended solids, organic compounds and nitrogen containing compounds. Low pH, high temperature, and no suspended solids, organic compounds etc will enhance the disinfecting effect.

Chlorine kills pathogens such as bacteria and viruses by replacement of hydrogen atoms by chlorine breaking the chemical bonds in their molecules. The molecule will the change shape or fall apart. As the enzymes are destroyed the pathogen will eventually die.

When chlorine is added to water, underchloric acids form and depending on the pH value, underchloric acid

partly expires to hypochlorite ions:
$Cl_2 + H_2O \rightarrow HOCl + H^+ + Cl^-$
$HOCl + H_2O \rightarrow H_3O^+ + OCl^-$

This falls apart to chlorine and oxygen atoms:
$OCl^- \rightarrow Cl^- + O$

Underchloric acid (HOCl) is more reactive and is a stronger disinfectant than hypochlorite (OCl^-). HOCl is split into hydrochloric acid (HCl) and atomar oxygen (O). So the disinfecting properties of chlorine in water are based on the oxidising power of free oxygen atoms and on chlorine substitution reactions.

The cell wall of pathogenic microorganisms is negatively charged by nature. As such, it can be penetrated by the neutral underchloric acid, rather than by the negatively charged hypochlorite ion. Underchloric acid can penetrate slime layers, cell walls and protective layers of microorganisms and effectively kills pathogens as a result. The microorganisms will either die or suffer from reproductive failure.

HOCl reacts faster and is more effective than OCl^-. The level of HOCl will decrease as the pH value goes up. The optimal pH for using chlorine with be at pH 5,5-7,5 (2).

Heat

Different combinations of temperture and time are able to inactivate microorganisms. When proteins are heated their three-dimensional structure will be destroyed as the weak hydrogen bonds dissociate due to the vibrations caused by heating. With destroyed proteins the microorganisms will not function properly.

Iodine

Iodine comes from the Greek word iodes meaning violet or purple. Elemental iodine, iodide or iodine from iodophors (iodine complexed with a solubilising agent that releases free iodine when in solution) are used for disinfection. The disinfecting ability of iodine is less influenced by the pH than chlorine as long as the pH is below 8-9. Iodine is widely used in the aquaculture industry for disinfection of eggs.

Ozonation

Ozone is a potent oxidant with bactericidal and virucidal abilities. Ozone decomposes rapidly to oxygen limiting the ability to maintain a sufficient residual ozone concentration for the necessary time period. Compared to freshwater more longlived reaction products are formed when brackish and seawater are ozoneated. The damaging effect of ozone is probably caused by changes in the membrane structure causing leakage of proteins and nucleic acids, as well as lipid oxidation (reviewed in 68).

Percolation

Percolation concerns the movement of fluids through porous materials, here the movement of wastewater through underground earth. The hope is, that this transport will withheld pathogenic microorganisms and in this way “disinfect” the wastewater.

Discussion

In the present Danish legislation "Bekendtgørelse nr. 755 af 08/07/2008 om autorisation og drift af akvakulturbrug og –virksomheder" (Ministerial order no. 755 of 08/07/2008 regarding authorisation and operation of aquaculture farms and – enterprises) fish cutting plants are obliged to percolate process wastewater. Dispensation from percolation of wastewater can be permitted if the wastewater is disinfected and the chosen method results in complete inactivation of infectious matters. A complete inactivation of pathogens will not be a realistic goal as this will require sterilization of the wastewater, which is not economical feasible for the industry. In order to reduce the risk of transfer of diseases to a tolerable level a lower intensity of inactivation is acceptable. In Norway the requirement is a 3 log reduction for the pathogens *Aeromonas salmonicida* subsp. s*almonicida* and ISAV. Under Danish conditions a 3 log reduction will probably also be appropriate in order to reduce the risks to an acceptable level.

In the scientific literature numbers are available to give an indication of the amount of virus that may be present in process wastewater and in fish offals and how little virus that can initiate an outbreak.

In seawater from Pacific herring confined for the production of spawn-on-kelp 700 pfu/ml VHSV was observed (38). In infection trials using Pacific herring up to $10^{7,7}$ pfu/g herring was detected at 6-8 days after infection. In the water in the flow-through aquarias at day 4-5, $10^{2,5}$ pfu/ml was obtained. When the water flow was turned off for 3 hours the water reached $10^{3,5}$ pfu/ml water. Virus shed by infected herring was on avereage > $10^{6,5}$ pfu/h/fish (62).
When groups of wild herring were confined in the laboratory, they experienced severe mortality, occasionally exceeding 50%, with the prevalence of VHSV reaching 100% by 14 d postcapture. At 7-21 d postcapture, VHSV titers peaked in excess of 10^8 pfu/g of tissue (63).

In rainbow trout infected with the freshwater isolate DK-3592B, the fish were positive at a low titer (7.1 x 10^2 $TCID_{50}$/g of tissue) by day 2 postchallenge, and the titer reached a peak (1.3 x 10^8 $TCID_{50}$/g of tissue) by day 7 (13).
Another infection trial reported mean titres of 5,3 x 10^6 $TCID_{50}$/g of tissue (29).

In experimentally infected rainbow trout challenged with VHSV by bath with 10^2, $10^{3,7}$, and 10^5 $TCID_{50}$/ml of the cumulative mortality was 44, 64, and 96%, respectively, at 14 d post infection (26).
In an experimental infection trial using the isolate J167 from the English outbreak in 2006, an infection dose as low as 10^1 $TCID_{50}$/ml water resulted in an accumulated mortality of 65% at day 21 in rainbow trout fry (19).
Vestergaard Jørgensen & Olesen reported that at the time when VHSV koncentration is highest in the fish (10^8 pfu/g of tissue), the amount of virus particles (pfu) in the water can be as high as 10^3 per ml of water (50). In infection trials at 10°C, the incubation phase was 1½ week when using a viral dose of 50 pfu/ml water. When the dose was lowered to 25 pfu/ml of water the incubation phase was extended to 6 weeks and at a dose of 10 pfu/ml, VHS was not observed during the next 6 months. The authorms comments this results by noting that 10 pfu/ml may be to low a dose under the circumstances used in the infection trial or that the incubation period may be longer than the 6 months, but that under other circumstances such a low dose will be able to initiate an outbreak (50).

These results show that the amount of VHSV can be quite high in tissue from VHSV infected fish during an outbreak, and a dose of virus as low as 10 $TCID_{50}$/ml water may be able to initiate an outbreak. The amount

of virus that can be found in water during an outbreak is 10^3-10^4 $TCID_{50}$/ml water. A 3 log reduction of virus will reduce the amount of VHSV to 0-10 $TCID_{50}$/ml water, a dose that probably only very seldom will be able to initiate a VHS outbreak.

In 11 of 15 wild-caught sockeye salmon in prespawning conditions IHNV was isolated from the following organs at a mean level among the positive organs (min – max) in pfu/g tissue of:
Gills 5,8 x 10^3 (3,0 x 10^2 - 5,5 x 10^5, 10 positive), Kidney 5,8 x 10^3 (1 positive), spleen 5,2 x 10^2 (1,0 x 10^2 – 1,3 x 10^3, 3 positive), pyloric caeca 5,1 x 10^2 (2,5 x 10^{1-} 1,4 x 10^4, 4 positive), Brain 5,0 x 10^1 (1 positive) and eggs 4,0 x 10^2 (1 positive). From fish in spawning conditions IHNV was isolated from nil to 100% of the fish within 2 weeks and virus incidens was high in all organs and fluids except brain and serum (77).
In a study of the possible role of waterborne IHNV in transmission of the disease among spawning sockeye salmon both infection rates and virus titres were higher in fish held at high density in a side channel than in fish in the adjacent river. Virus was never isolated from river water, but was found in water from the side channel at levels ranging from 32.5 to 1600 pfu/ml (78).
In rainbow trout the amount of IHNV i ovarian fluid ranged from 10^1 – $10^{6,5}$ $TCID_{50}$/ml (7).
In an infection trial in rainbow trout using IHNV mean titres of 5,1 x 10^5 $TCID_{50}$/g of tissue was reported (29).
As these values are correspondable to the VHSV values a 3 log reduction will also be acceptable for IHNV.

For IPNV Wolf & Quimby (unpublished, in 101) reported average IPNV titer in five adult carrier brook trout in $TCID_{50}$/g tissue ranging from $10^{0,3}$ in muscle to $10^{6,7}$ in kidney. In an IPNV infection trial in brook trout virus was shedded in the feces 8 weeks post infection at a mean titre of $10^{3,5-4}$ $TCID_{50}$/g (12). In another infection trial using IPNV Sp, moribund rainbow trout alevins kept at 16°C had a titer of 10^4 – 10^6 pfu/g fish, whereas alevins kept at 10°C which had a titer up to 10^8 pfu/g (21).
In a hatchery outbreak, a level of $10^{4,4}$ infective particles per ml in a tank supplied with 88 l/min of water was measured (101). Desautels & MacKelvie (17) titrated water from three troughs of trout fry during a serious IPN epizootic in a commercial rearing establishment at found an excess of 10^5 $TCID_{50}$/ml.
It is assumed that the amount of virus in process water will be less that the amount found in water during an IPN outbreak, and as such a 3 log reduction will also for this virus reduce the amount to an acceptable risk, regarded the water is not released to watersheds where IPNV free farms are situated downstream.

In Norway at present and in the historic Danish legislation a number of different methods are/were approved for sanitazion of wastewater. These included treatment with

pH (acidic):
Mechanic separation (≤ 300 µm filter) followed by acid treatment to pH ≤ 3.0 for ≥ 8 hours.

The literature review showed that VHSV, IHNV and several other viruses are not inactivated by treatment at a pH of 4 for 24 hours. In order to decimate VHSV and IHNV to a non detectable level a treatment of pH 3 for 3-4 hours is needed. This will also inactivate *Aeromonas salmonicida* and probably also salmonid alphavirus and ISAV. *Yersinia ruckeri* will be decimated to some degree by this treatment, but not necessarily 3 log. Nodavirus is extremely acid stable and will not be inactivated by acid conditions. IPNV is also very stable at low pH, but pH < 2 should be able to inactivate IPNV (as well as *Yersinia* ruckeri) although survival for 35 days at pH 2 has been reported. Ranavirus has been recorded to both survive and be inactivated at pH 4.

pH (basic):
Mechanic separation (≤ 300 µm filter) followed by basic treatment to pH ≥ 12.0 for ≥ 24 hours.

VHSV, IHNV, IPNV, SVCV, PFRV, SAV and *Aeromonas salmonicida* are inactivated at pH 12 for 24 hours. Nodavirus has been reported inactivated at that pH but also to survive. *Yersinia ruckeri* is also difficult to inactivate at pH 12, but will be decimated.
Ranavirus has been recorded to both survive and be inactivated at pH 12.

UV-irradiation:
a) chemical precipitation (Fe- and/or Al-salts) followed by UV irradiation of the supernatant using an UV-dose ≥ 25 mWs/cm^2.
b) Mechanic separation (≤ 40 µm filter) followed by UV irradiation of the supernatant using an UV-dose ≥ 25 mWs/cm^2.

In laboratory trials a dose of 25 mWs/cm^2 (254 nm) induces satisfactory decimations of VHSV, IHNV and ISAV. In laboratory trial using wastewater from a fish cutting plant 3,1 mWs/cm^2 was needed to decimate VHSV 3 log. For IHNV 4 mWs/cm^2 was needed for a 3 log reduction in laboratory trials. For ISAV the needed dose for a 3 log reduction was 7,5 mWs/cm^2. In infection trial using tissue homogenate from ISA infected fish, a dose of 20 mWs/cm^2 was needed to decimate the virus so much that ISA was not induced in the IP injected fish. The bacteria *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio anguillarum* and *Yersinia ruckeri* a 3 log decimation was obtained using a dose of 5 – 25 mWs/cm^2 in laboratory trials. For *Y. ruckeri*, in full scale trials using wastewater from fish slaugtherhouses a dose of 250 mWs/cm^2 gave a reduction of only 1 log despite precipitation with ferrichlorid; 2½ log was obtained using a dose of 1200 mWs/cm^2 and prefiltration with a 20 µm filter.
IPNV is far more resistant to UV light than VHSV and IHNV. In laboratory trials a dose of 200 – 250 mWs/cm^2 was required to obtain a 3 log reduction, and 800 mWs/cm^2 was needed for a 6 log reduction. In full scale trials using wastewater from fish slaugtherhouses a dose of 250 mWs/cm^2 produced only a ½ - 1 log reduction in virus titer. In order not to detect IPNV anymore a dose of 1500 mWs/cm^2 was needed.
Nodavirus also seems to be quite resistant to UV irradiation. In laboratory trials a dose of 100 – 211 mWs/cm^2 has been reported to induce a 3 log reduction. In an infection trial a dose of 100 mWs/cm^2 of the virus was reported to inhibit disease in the fish.
The results from the full scale trials suggest that even though UV irradiation in laboratory trials is effective it may not be possible to use this method on process wastewater in fish slaugtherhouses/cutting plants, despite pretreatment of the water by filtration or chemical precipitation. The authors conclude though that the ineffectual pretreatment probably was due to operating problems and inadequate optimisation of the process. Further full-scale tests showed that the quality of the wastewater was improved by chemical precipitation, and the best result was obtained by first adding ferrichlorid to pH 3,9 followed by addition of NaOH to pH 6,4, polymerisation and flotation. This treatment reduced the amount of organic matter with 65% measured as COD (chemical oxygen demand) and reduced the amount of total nitrogen with 73%. Furthermore was fat and floating material separated in the flotation tank (28). Whether UV irradiation after this treatment whould provide an acceptable reduction of the pathogens is unknown but probable.

Chlorination:

a) mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 10 mg/l residual chlorine after 15 minutes treatment.

b) mechanic separation (≤ 300 µm filter) or chemical precipitation (Fe- and/or Al-salts) followed by chlorination of the supernatant using an initial concentration of ≥ 50 mg/l residual chlorine and ≥ 2 mg/l residual chlorine after 25 minutes treatment.

Generally speaking the amount of chlorine needed depends on the temperature, the pH, the degree of organic contamination and the titer of the pathogen. The necessary amount will rise if the temperature fall, the pH rise, more dirty conditions prevail and the titer of the pathogen goes up. The dose mentioned in the Danish ministerial order no 755 of 28/07/2005 and in the Norwegian list of approved methods (50 mg/l free chlorine (10 mg/l residual chlorine efter 15 min or 2 mg/l residual chlorine after 25 min) is probably acceptable for a 3 log reduction under clean conditions for VHSV, IHNV and bacterial pathogens, and also for the more resistant viruses such as IPNV and nodavirus. But for the conditions that prevail in wastewater from fish cutting plants this dose will not be acceptable without a proper pretreatment of the water. For *Yersinia ruckeri* the dose to induce a 3 log reduction is 250 mg/l for more than 2 hours in full-scale tests using wastewater from fish cutting plants. In a full-scale test using NaOCl IPNV was stable at the same dose administered for 1 hour, whereas when the dose was administered as chloramine-T a 4 log reduction was obtained.

In a full-scale trial where the wastewater was pretreated by adding NaOH to pH 12 followed by addition of ferrichlorid to pH 6,5-7,5 *Y. ruckeri* was inactivated using 48 mg/l chlorine for half an hour. This result shows that chemical precipitation of the wastewater will reduce the amount of chlorine needed to disinfect the wastewater. Mechanical separation was not tested.

Pretreatment of the wastewater was obligatory in the historic Danish ministerial order either as mechanic separation using a filter or as chemical precipitation.

A few papers have reported on the use of chlorine produced by electrolyzation of the water, which seems a usable method.

Heat treatment:

a) 65°C for 10 minutes.
b) 70°C for 5 minutes.
c) 75°C for 4 minutes.
d) 80°C for 3 minutes.
e) 85°C for 2 minutes.
f) 90°C for 1 minute.
g) 95°C for 45 seconds.
h) 100°C for 30 seconds.

Despite the conflicting results reported by different authors or obtained from different experiments the combinations of time and temperature stated in the Danish ministerial order no 755 of 28/07/2005 and in the Norwegian list of approved methods will probably be acceptable for fish pathogenic bacteria and viruses for at least a 3 log reduction if a proper stirring of the wastewater is secured to avoid pockets of water not reaching the desired temperature for the stated amount of time.

Percolation

It has not been possible to find any references describing the decimating effect of percolating of fish pathogenic viruses. Furthermore it has not been possible to find publications describing the effect of percolating other birnaviruses. It is therefore not possible to validate if this procedure is safe to use.

Iodine products

Iodine based disinfection products are useful for disinfection of virus and bacterias but less suitable for use when parasites and fungi are the microorganism in question. IPNV and nodavirus seems to be a bit more resistant that VHSV and IHNV. It has not been possible to find published tests on the efficiency of iodine using wastewater from fish slaugtherhourse or cutting plants. Iodine is sensitive towards titer of pathogen, Temperature, pH and organic contamination, with more iodine needed as the titer of the pathogen goes up, temperature goes down, pH ≥ 8 and organic contamination goes up. Recommeded dose: ≥ 150 ppm for 10 min at pH < 8.

Ozone

Ozone seems to be an effective product to decimate the concentration of fish viral and bacterial pathogens. None of the papers papers covered in this report has tested ozone under circumstances comparable to the conditions prevailing in wastewater from fish cutting plants and it is as such unknown how usable the method will be for this specific purpose. In Norway ozone is not listed as an approved method for disinfection of wastewater from fish slaugtherhouses/cutting plants, but the method is approved for use in infection trial facilities with the following doses:

a) freshwater: ≥ 15 mg/l residual ozone after 15 minutes treatment (corresponds to a C T value of 135 mg*s/l).
b) sea water: ≥ 0,2 mg/l TRO (total residual oxidants) after 15 minutes treatment (corresponds to a C T value of 180 mg*s/l).

If these doses are adopted, and the water is pretreated, all fish pathogenic bacteria and viruses should be inactivated.

Pretreatment of water

Regardless of the method chosen for disinfection of the wastewater from fish processing plants the effect will be better the cleaner the water is. It is therefore very important the the water is treated to reduce the amount of organic matter in the water. The results from the full scale trials in Norway referred to under the headline "Chlorination" in this paragraph shows the importancy of pretreatment of the water before disinfection. There are several ways of pretreating the water of which mechanical separation and chemical precipitation was accepted in the historical Danish legislation.

Tables

pH

Virus

VHSV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
4,0 x 10^7 pfu/ml	HCl	10 min		2,5	8-10	Survival		52	Final concentration 1,5 x 10^4
4,0 x 10^7 pfu/ml	HCl	60 min		2,5	8-10	”inactivated”		52	Final concentration < 10
$10^{5,2}$ $TCID_{50}$/ml	pH	60 min		3		Survival		4	$10^{2,2}$ $TCID_{50}$/ml after 60 min
$10^{5,2}$ $TCID_{50}$/ml	pH	180 min		3		”inactivated”		4	
$10^{5,8}$ $TCID_{50}$/ml	pH	60 min		3		99,9 % reduction	5 % calf serum added	4	<1
$10^{5,8}$ $TCID_{50}$/ml	pH	180 min		3		”inactivated”	5 % calf serum added	4	
$10^{5,1}$ $TCID_{50}$/ml	HCl	24 hours		4	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: $10^{2,2}$ $TCID_{50}$/ml
	pH	7 days		4		Survival		20, Dixon (pers. com.)	
$10^{6,2}$ $TCID_{50}$/ml	pH	60 min		9	10	Stable		4	
$10^{5,7}$ $TCID_{50}$/ml	KOH	48 hours		11	4	Stable	Pathogen mixed into minced herring	47	
$10^{5,7}$ $TCID_{50}$/ml	KOH	1 t		12	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: $10^{2,2}$ $TCID_{50}$/ml
	pH	6 hours		12		inaktiveret		20, Dixon (pers. com.)	
1,5 x 10^7 pfu/ml	NaOH	120 min		12,2	8-10	Survival		52	Final concentration 1,8 x 10^4
$10^{6,5}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		> 99,99 % reduction	10 % calf serum added	5, 4	Survival
$10^{5,8}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”		4	
$10^{5,8}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
$10^{6,5}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”		4	

Conclusion: VHSV is inactivated at pH 3 and pH 12 after 3 hours contact time. For 3 log inactivation pH 3 or pH 12 for 10 minuts is suitable.

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^6 TCID_{50}$/ml	pH	4 hours		3	4	Not detectable	Virus in MEM	79	
	citrate/phos-phate buffer	7 hours		4,0	22	Survival		100	
	pH	7 days		4		Survival		20, Dixon (pers. com.)	
	pH	6 hours		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: IHNV is inactivated at pH 3 for 4 hours, but not at pH 4. At pH 12, tested after 6 hours contact time, IHNV is inactivated.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Formic acid	8 hours	-	4	-	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Testet at pH 3,5, 4,0 and 4,5 at 8 hours and 24 hours.
	pH	7 days		4		”inactivated”		20, Dixon (pers. com.)	
	pH	30 min		4		Not detectable	Addition of HCl or NaOH to virus in L15 medium to pH 3, 4, 5, 7, 9 or 11.	27	
	pH	30 min		5-9		Stable	Addition of HCl or NaOH to virus in L15 medium to pH 3, 4, 5, 7, 9 or 11.	27	
	pH	30 min		11		> 90% reduction	Addition of HCl or NaOH to virus in L15 medium to pH 3, 4, 5, 7, 9 or 11.	27	
	NaOH	48 hours	-	11,5	-	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Testet at pH 11,0, 11,5 and 12,0 at 8, 12, 24 and 48 hours.
	NaOH	24 hours	-	12	-	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Testet at pH 11,0, 11,5 and 12,0 at 8, 12, 24 and 48 hours.
	pH	24 hours		12		Survival		20, Dixon (pers. com.)	

Conclusion: ISAV is inactivated at pH 4, but the contact time has to be relative long. There is disagreement among the references regarding inactivation at pH 12.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,6}$ $TCID_{50}$/ml	Formic acid (HCOOH)	6 min		1,5	7	Not detectable (> 5 log reduction)	Laboratory trial, 1 part process water + 2 parts "bløggevand" from fish slaugtherhouse.	28	
$10^{5,5}$ $TCID_{50}$/ml	Formic acid (HCOOH)	1 t		2,0		Not detectable (> 4 log reduction)	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, "bløggevand" (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{7,0}$ $TCID_{50}$/ml	pH	35 days		2	4	5 log reduction		74	Isolate VR-299
$10^{7,0}$ $TCID_{50}$/ml	pH	20 days		2	4	3 log reduction		74	Isolate VR-299. Result read on a graph.
6,7 x 10^{6} pfu/ml	HCl	60 min		2,5	8-10	Stable		52	Type Sp. Final concentration 5,3 x 10^{6}
$10^{5,5}$ $TCID_{50}$/ml	Formic acid (HCOOH)	24 hours		2,5		1-2 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, "bløggevand" (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{6,6}$ $TCID_{50}$/ml	Formic acid (HCOOH)	10 hours		2,5	7	2 log reduction	Laboratory trial, 1 part process water + 2 parts "bløggevand" from fish slaugtherhouse.	28	
$10^{5,5}$ $TCID_{50}$/ml	Formic acid (HCOOH)	24 hours		3		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, "bløggevand" (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{7,5}$ $TCID_{50}$/ml	pH	4 hours		3	4	Stable	Virus in MEM	171	
$10^{6,5}$ $TCID_{50}$/ml	pH	360 min		3		Stable		4	
$10^{6,8}$ $TCID_{50}$/ml	pH	360 min		3		Stable	5 % calf serum added	4	
$10^{6,6}$ $TCID_{50}$/ml	Formic acid (HCOOH)	10 hours		3,5	7	3 log reduction	Laboratory trial, 1 part process water + 2 parts "bløggevand" from fish slaugtherhouse.	28	
	Citrate/phos-phate buffer	14 days		4,0	22	Survival		100	
	pH	28 days		4		Survival		20, Dixon (pers. com.)	

$10^{7,0}$ $TCID_{50}$/ml	pH	35 days		7	4	Stable		74	Isolate VR-299
$10^{6,5-7,0}$ $TCID_{50}$/ml	PBS	109 uger			4	Survival		74	Isolate VR-299
$10^{5,8}$ $TCID_{50}$/ml	pH	60 min		9	10	Stable		4	
$10^{7,0}$ $TCID_{50}$/ml	pH	35 days		9	4	5 log reduction		74	Isolate VR-299
$10^{7,0}$ $TCID_{50}$/ml	pH	26 days		9	4	3 log reduction		74	Isolate VR-299. Result Read off a graph.
$10^{5,3}$ $TCID_{50}$/ml	KOH	48 hours		10	4	Stable	Pathogen mixed into minced herring	47	
$10^{5,3}$ $TCID_{50}$/ml	KOH	24 hours		11	4	Survival	Pathogen mixed into minced herring	47	
$10^{5,3}$ $TCID_{50}$/ml	KOH	48 hours		11	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: 10^2 $TCID_{50}$/ml
$10^{5,5}$ $TCID_{50}$/ml	NaOH	24 hours		11,6		3½ log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,5}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
$10^{4,8}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”		4, 5	
$10^{4,5}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
$10^{4,8}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”		4	
$10^{5,5}$ $TCID_{50}$/ml	NaOH	24 hours		12.0		3½ log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{5,3}$ $TCID_{50}$/ml	KOH	16 hours		12	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: 10^2 $TCID_{50}$/ml
$10^{6,6}$ $TCID_{50}$/ml	NaOH	6 min		12,0	7	Not detectable (> 5 log reduction)	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
	pH	20 min		12		”inactivated”		20, Dixon (pers. com.)	
2,2 x 10^4 pfu/ml	NaOH	10 min		12,2	8-10	”inactivated”		52	Final concentration < 10
$10^{5,5}$ $TCID_{50}$/ml	NaOH	1 hour		12,4		Not detectable (> 4 log reduction)	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.

Conslution: In order to secure inactivation of IPNV, the pH has to as low as 2 or high as 12 with a contact time for at least 1 hour. There are reports stating surval time of several weeks at pH 2, but in full scale trials using wastewater containing blood, slime and skin scrapings in saltwater 1 hour contact time was sufficient to inactivate IPNV.

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,5}$ $TCID_{50}$/ml	HCl	7 days		2	15	Stable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,5}$ $TCID_{50}$/ml	HCl	21 days		2	15	Survival (5 log reduction)	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,5}$ $TCID_{50}$/ml	HCl	42 days		2	15	Not detectable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
10 µg purified virus	HCl	10 min		3	20	Not inactivated(0/800 larvae survived, control 472/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
$10^{6,5}$ $TCID_{50}$/ml	HCl	42 days		3	15	Stable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
	pH	7 days		4		Survival		20, Dixon (pers. com.)	
10 µg purified virus	PBS	10 min		7	20	Not inactivated(0/800 larvae survived, control 472/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
$10^{6,5}$ $TCID_{50}$/ml	NaOH	42 days		9	15	Stable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,0}$ $TCID_{50}$/ml	KOH	48 hours		10	4	Stable	Pathogen mixed into minced herring	47	
$10^{6,0}$ $TCID_{50}$/ml	KOH	24 hours		11	4	Survival	Pathogen mixed into minced herring	47	

$10^{6,0}$ $TCID_{50}$/ml	KOH	48 hours		11	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: 10^2 $TCID_{50}$/ml
$10^{6,5}$ $TCID_{50}$/ml	NaOH	7 days		11	15	Survival (2-3 log reduction)	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,5}$ $TCID_{50}$/ml	NaOH	15 days		11	15	Not detectable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,5}$ $TCID_{50}$/ml	NaOH	15 days		11	15	Not detectable	Grown virus diluted 1:100 in distilled water. Tested after 1 hour and 1, 3, 7, 15, 21 and 42 days.	30	Isolate: sea bass nodavirus
$10^{6,0}$ $TCID_{50}$/ml	KOH	12 hours		12	4	Not detectable	Pathogen mixed into minced herring	47	Detection limit: 10^2 $TCID_{50}$/ml
10 µg purified virus	NaOH	10 min		12	20	”Effective” (238/800 larvae survived, antigen ELISA negativ - control 472/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
	pH	24 hours		12		Survival		20, Dixon (pers. com.)	

Conclusion: Nodavirus seemingly is able to withstand low pH. There are disagreements conserning the ability of the virus to withstand pH 12.

PFRV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
6,5 log10 $TCID_{50}$/ml	pH	60 min		9	10	Stable		4	
6,5 log10 $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
6,8 log10 $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”		4, 5	
6,8 log10 $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
6,2 log10 $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”		4	

Conclusion: PFRV is inactivated at pH 12 for 5 minuts.

Ranavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pH	28 days		4		Survival		20, Dixon (pers. com.)	
	pH	1 hour		4		Not detectable	Virus in cell culture medium.	67	Isolat: EHNV
	pH	6 hours		12		Survival		20, Dixon (pers. com.)	
	pH	1 hour		12		Not detectable	Virus in cell culture medium.	67	Isolat: EHNV

Conclusion: the literature does not agree on the inactivation of ranavirus at pH 4 and 12.

SAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 $TCID_{50}$/ml	pH	4 hours		3	4	Not detectable	Virus in MEM	79	Salmon pancreas disease virus (SPDV)
	HCl	mixing		4	4	”inactivated”		33	SAV1
	Formic acid	5 min		4	4	Survival. 99,99 % reduction		33	SAV1
	Formic acid	1 dag		4	4	”inactivated”	Tested after 5 min, 1 day and 7 days.	33	SAV1
	Formic acid	7 days		5	4	”inactivated”	Tested after 5 min, 1 day and 7 days.	33	SAV1
	Formic acid	7 days		6	4	Stable	Tested after 5 min, 1 day and 7 days.	33	SAV1
	Formic acid	7 days		7,2	4	Stable	Tested after 5 min, 1 day and 7 days.	33	SAV1
	NaOH	mixing		12	4	”inactivated”		33	SAV1

Conclusion: SAV can be inactivated at pH 4 using a contact time of 24 hours or > 3 log reduction after a contact time of 5 min. At pH 3 or 12 hourshe virus is inactivated immediately.

SVCV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{7,2}$ $TCID_{50}$/ml	pH	30 min		3	room temp.	> 3 log reduction	Titrated after 30, 60 and 120 minuts.	3	
$10^{7,2}$ $TCID_{50}$/ml	pH	120 min		3	room temp.	6 log reduction	Titrated after 30, 60 and 120 minuts.	3	
	pH	28 days		4		Survival		20, Dixon (pers. com.)	
$10^{6,5}$ $TCID_{50}$/ml	pH	60 min		9	10	Stable		4	

	pH	6 days				Survival		64	
$10^{7,2}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
$10^{6,8}$ $TCID_{50}$/ml	NaOH	5 min	2%	11,85-11,90		> 99,99 % reduction		4, 5	Survival
$10^{7,5}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”	10 % calf serum added	4	
$10^{7,5}$ $TCID_{50}$/ml	NaOH	10 min	2%	11,85-11,90		”inactivated”		4	
	pH	6 hours		12		Survival/”inactivated”		20, Dixon (pers. com.)	

Conclusion: SVCV kan inaktiveres ved pH 3 (3 log, 30 min) og pH 12.

Bacteria

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,4 x 10^7 cfu/ml	citrate/phos-phate buffer	90 min		4,0	22	”inactivated”		100	
	pH	2 hours		4		”inactivated”		20, Dixon (pers. com.)	
5 x 10^8 cfu/ml	KOH	12 hours		10	22	”inactivated”	Pathogen mixed into minced herring	47	Testet efter 12 hours, 24 hours og 48 hours
	pH	10 min		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: *A. salmonicida* is inactivated at pH 4 (testet at 90 min contact time) and pH 12 (10 min contact time).

Lactococcus garviae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pH	7 days		4		Survival		20, Dixon (pers. com.)	
	pH	14 days		12		Survival		20, Dixon (pers. com.)	

Conclusion: According to this experiment *L. garviae* can withstand both pH 4 and 12.

Listonella (Vibrio) anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pH	24 hours		4		Survival		20, Dixon (pers. com.)	
	pH	30 min		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: *V. anguillarum* can withstand pH 4, but is inactivated at pH 12 using a contact time of 30 min.

Mycobacterium chelonae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,4 x 10^7 cfu/ml	Citrate/phos-phate buffer	> 14 days		4,0	22	”inactivated”		100	
	pH	2 days		4		Survival		20, Dixon (pers. com.)	
	pH	48 hours		12		Survival		20, Dixon (pers. com.)	

Conclusion: *M. chelonae* can withstand both pH 4 and 12.

Photobacterium damselae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pH	24 hours		4		Survival		20, Dixon (pers. com.)	
	pH	10 min		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: *P. damselae* survives pH 4, but is inactivated at pH 12 for 10 min.

Renibacterium salmoninarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,4 x 10^7 cfu/ml	Citrate/phos-phate buffer	4 hours		4,0	22	Survival		100	
	pH	24 hours		4		”inactivated”		20, Dixon (pers. com.)	
	pH	6 hours		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: *R. salmoninarum* survives pH 4 for at least 4 hours, but is inactivated after 24 hours. Can be inactivated at pH 12 using a contact time of 6 hours.

Streptococcus iniae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pH	24 hours		4		”inactivated”		20, Dixon (pers. com.)	
	pH	30 min		12		”inactivated”		20, Dixon (pers. com.)	

Conclusion: *S. iniae* can be inactivated at pH 4 (24 hours contact time) og pH 12 (30 min contact time).

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{8,28}$ cfu/ml	Formic acid (HCOOH)	1 hour		1,5	7	Not detectable (> 8 log reduction)	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{8,28}$ cfu/ml	Formic acid (HCOOH)	6 min		1,5	7	7 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{4,69}$ cfu/ml	Formic acid (HCOOH)	10 hours		1,98	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{6,77}$ cfu/ml	Formic acid (HCOOH)	0,1 hour		2		Not detectable (> 6 log reduction)	Full-scale trial, wastewater from fish slaugtherhouses.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{6,77}$ cfu/ml	Formic acid (HCOOH)	1 hour		2,5		Not detectable (> 6 log reduction)	Full-scale trial, wastewater from fish slaugtherhouses.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	Formic acid (HCOOH)	10 hours		2,5	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{8,28}$ cfu/ml	Formic acid (HCOOH)	5 hours		2,5	7	Not detectable (> 8 log reduction)	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	

$10^{6,77}$ cfu/ml	Formic acid (HCOOH)	24 hours		3		4 log reduction	Full-scale trial, wastewater from fish slaugtherhouses.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{8,28}$ cfu/ml	Formic acid (HCOOH)	10 hours		3,5	7	3 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{4,69}$ cfu/ml	Formic acid (HCOOH)	10 hours		3,98	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
	pH	24 hours		4		Survival		20, Dixon (pers. com.)	
$10^{6,77}$ cfu/ml	NaOH	24 hours		11,6		2-3 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{6,77}$ cfu/ml	NaOH	5 hours		12,0		Not detectable (> 6 log reduction)	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{8,28}$ cfu/ml	NaOH	10 hours		12,0	7	Stable? Survival.	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
	pH	10 min		12		”inactivated”		20, Dixon (pers. com.)	
$10^{6,77}$ cfu/ml	NaOH	1 hour		12,4		Not detectable (> 6 log reduction)	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	NaOH	24 hours		12,44	7	2 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{8,28}$ cfu/ml	NaOH	10 hours		12,5	7	2-3 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{4,69}$ cfu/ml	NaOH	5 hours		12,70	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{4,69}$ cfu/ml	NaOH	10 hours		12,81	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish	28	Salinity 14-15 ‰

							slaugtherhouse.		
$10^{8,28}$ cfu/ml	NaOH	10 hours		13,0	7	3-4 log reduction	Laboratory trial, 1 part process water + 2 parts "bløggevand" from fish slaugtherhouse.	28	

Conclusion: *Y. ruckeri* can be inactivated at pH 2.5 (contact time 1-10 hours). There are reports of 3 log inactivation at pH 3.5 and a contact time of 10 hours. There are conflicting data with regard to high alkaline pH, in full scale trials using wastewater from slaughterhouses complete inactivation at pH> 12 as well as only 2-4 log reduction is shown. This may be due to lacking proper stirring in the full-scale experiments so pockets where the bacteria have not been treated have occurred.

Parasites

Pathogen	Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
Trichodina jadranica	2,1	HCl	24 hours		5	25	Stable (2,4)	*In vivo*, eel	75	Categorisation (category/number of parasites): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000
Gyrodactylus salaris		pH	Few days		< 5		Dies		1	No reference stated!
Myxosoma cerebralis		KOH	2 days	0,5%		22	All dead	*In vitro* - spores	42	Tested at 0,01, 0,1 and 1%

UV

Virus

VHSV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^{4-5} $TCID_{50}$/ml	UV		0,79 ± 0,15 mWs/cm^2			99,9% reduction	Virus in fresh water.	81	UV-C
10^{4-5} $TCID_{50}$/ml	UV		app. 1,5 mWs/cm^2			"inactivated"	Virus in fresh water.	81	Read off a graph.
app. 6 log TCID	UV		1,8 mWs/cm^2			99,9% reduction		46	UV-C
$10^{7,2}$ $TCID_{50}$/ml	UV	10 min	254 nm, 5 cm afstand		20	"inactivated"		4	
10^{4-5} $TCID_{50}$/ml	UV		3,1 ± 0,18 mWs/cm^2			99,9% reduction	Virus in wastewater from fish cutting plant.	81	UV-C
app. 6 log TCID	UV		4,0 mWs/cm^2			"inactivated"		46	UV-C
10^{4-5} $TCID_{50}$/ml	UV		app. 4 mWs/cm^2			"inactivated"	Virus in wastewater from	81	Read off a graph.

							fish cutting plant.		
	UV		10 mWs/cm^2			LD90		76	

Conclusion: VHSV is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 4 mWs/cm^2. It has been reported though, that 10 mWs/cm^2 is needed for a 2 log reduction.

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		1 mWs/cm^2			99% infectivity reduction		102	
$10^{4,8-6,3}$ $TCID_{50}$/ml	UV		1,0 - 2,0 mWs/cm^2			ID_{99}	UV intensity: 100 µW/cm^2	103	strain CHAB
$10^{6,8-7,8}$ $TCID_{50}$/ml	UV		1,5 - 3,0 mWs/cm^2			ID_{99}	UV intensity: 200 µW/cm^2	103	strain RTTO
	UV		2 - 3 mWs/cm^2			99% reduction		104	
	UV		2 mWs/cm^2			3 log reduction		86	
$10^{4,8-6,3}$ $TCID_{50}$/ml	UV	30 sec	3 mWs/cm^2			>2-4 log reduction	UV intensity: 100 µW/cm^2	103	strain CHAB
$10^{6,8-7,8}$ $TCID_{50}$/ml	UV	30 sec	4 mWs/cm^2			>3 log reduction	UV intensity: 200 µW/cm^2	103	strain RTTO

Conclusion: IHNV is susceptible to UV irradiation with 3 log reduction when exposed to 4 mWs/cm^2.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^{4-5} $TCID_{50}$/ml	UV		3,3 ± 0,35 mWs/cm^2			99,9% reduction	Virus in fresh water.	81	UV-C
10^{4-5} $TCID_{50}$/ml	UV		5,1 ± 1,3 mWs/cm^2			99,9% reduction	virus i havvand	81	UV-C
10^{4-5} $TCID_{50}$/ml	UV		app. 5,5 mWs/cm^2			”inactivated”	Virus in fresh water.	81	Read off a graph.
10^{4-5} $TCID_{50}$/ml	UV		app. 6,5 mWs/cm^2			”inactivated”	virus i havvand	81	Read off a graph.
10^{4-5} $TCID_{50}$/ml	UV		7,2 ± 1,6 mWs/cm^2			99,9% reduction	Virus in wastewater from fish cutting plant.	81	UV-C
	UV		7,5 mWs/cm^2	7,9	5	99,9% reduction	Sea water, sterile filtered.	73	UV-C
10^{4-5} $TCID_{50}$/ml	UV		app. 8 mWs/cm^2			”inactivated”	Virus in wastewater from fish cutting plant.	81	Read off a graph.
	UV	-	20 mWs/cm^2	-	-	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Tested at UV-doses 0,5 - 50 mWs/cm^2.

Conclusion: ISAV is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 8 mWs/cm^2.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{7,3-8,3}$ $TCID_{50}$/ml	UV		100 - 150 mWs/cm^2			ID_{99}	UV intensity: 1000 µW/cm^2	103	serotype Buhl
	UV		100 mWs/cm^2			99% infectivity reduction		102	
$10^{6,7}$ $TCID_{50}$/ml	UV		118,8 ± 5,7 mWs/cm^2			99,9% reduction	Virus in fresh water.	81	UV-C
	UV		150 mWs/cm^2			99% reduction		104	Type buhl
$10^{6,7}$ $TCID_{50}$/ml	UV		app. 170 mWs/cm^2			”inactivated”	Virus in fresh water.	81	Read off a graph.
$10^{7,3-8,3}$ $TCID_{50}$/ml	UV	3 min 20 sec	200 mWs/cm^2			3 log reduction	UV intensity: 1000 µW/cm^2	103	serotype Buhl
	UV		200 mWs/cm^2			3 log reduction		86	
	UV		246 mWs/cm^2	7,9	5	99,9% reduction	Sea water, sterile filtered.	73	UV-C
$10^{4,5}$ $TCID_{50}$/ml	UV, after ferrichlorid-precipitation		250 mWs/cm^2		75	½-1 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{8,2}$ $TCID_{50}$/ml	UV	60 min	254 nm, 5 cm afstand		20	”inactivated”		4	
	UV		330 mWs/cm^2			LD80		76	
10^{3-4} $TCID_{50}$/ml	UV		336,7 ± 27,5 mWs/cm^2			99,9% reduction	Virus in wastewater from fish cutting plant.	81	UV-C
$10^{7,0}$ $TCID_{50}$/ml	UV	6-15 min	720 – 1800 mWs/cm^2			6 log reduction	2000 µW/cm^2	74	Isolate VR-299. Result read off a graph.
$10^{7,0}$ $TCID_{50}$/ml	UV	30 min	792 mWs/cm^2			5,8 log reduction	440 µW/cm^2	74	Isolate VR-299. Result read off a graph.
10^{3-4} $TCID_{50}$/ml	UV		app. 1500 mWs/cm^2			”inactivated”	Virus in wastewater from fish cutting plant.	81	Read off a graph.

Conclusion: IPNV is susceptible to UV irradiation with 3 log reduction when exposed to 250-350 mWs/cm^2.

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10 µg purified virus	UV		10 mWs/cm^2	7	20	Not inactivated(0/800 larvae survived, control 326/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
	UV		100 mWs/cm^2			99% infectivity reduction		102	Tvivl om referencens pålidelighed
10 µg purified virus	UV		100 mWs/cm^2	7	20	"Effective" (222/800 larvae survived, antigen ELISA negative - control 326/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
	UV		104 mWs/cm^2	7,9	5	99,9% reduction	Sea water, sterile filtered.	73	UV-C
	UV	8 min	211,2 mWs/cm^2			3 log reduction	440 µW/cm2	30	Isolate: sea bass nodavirus
	UV	10 min	264 mWs/cm^2			Not detectable	440 µW/cm2	30	Isolate: sea bass nodavirus

Conclusion: Nodavirus is susceptible to UV irradiation with 3 log reduction when exposed to 100-200 mWs/cm^2.

PFRV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		1 mWs/cm^2			99% infectivity reduction		102	
$10^{5,3}$ $TCID_{50}$/ml	UV	10 min	254 nm, 5 cm afstand		20	”inactivated”		4	

SVCV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		1 mWs/cm^2			99% infectivity reduction		102	Tvivl om referencens pålidelighed
$10^{5,2}$ $TCID_{50}$/ml	UV	10 min	254 nm, 5 cm afstand		20	”inactivated”		4	

Viruses from eel

Pathogen	Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
EVA		UV		1 mWs/cm^2			99% infectivity reduction		102	
EVEX		UV		1 mWs/cm^2			99% infectivity reduction		102	

Conclusion: EVA and EVEX are susceptible to UV irradiation with 2 log reduction when exposed to 1 mWs/cm^2.

Channel catfish virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		2 mWs/cm^2			99% reduction		104	
$10^{6,55\text{-}7,05}$ $TCID_{50}$/ml	UV		1,8 - 2,0 mWs/cm^2			ID_{99}	UV intensity: 100 µW/cm^2	103	

Conclusion: CCV is susceptible to UV irradiation with 2 log reduction when exposed to 2 mWs/cm^2.

OMV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		2 mWs/cm^2			99% reduction		104	
$10^{3,05}$-$10^{6,55}$ $TCID_{50}$/ml	UV		1,0-2,0 mWs/cm^2			ID_{99}	UV intensity: 100 µW/cm^2	103	
	UV		1,4 mWs/cm^2			3 log reduction		86	

Conclusion: OMV is susceptible to UV irradiation with 2 log reduction when exposed to 2 mWs/cm^2.

Chum salmon virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{4,3}$-$10^{5,05}$ $TCID_{50}$/ml	UV		100 mWs/cm^2			99% reduction	UV intensity: 1000 µW/cm^2	103, 104	

Herpesvirus salmonis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{4,05}$-$10^{4,30}$ $TCID_{50}$/ml	UV		2 mWs/cm^2			99% reduction	UV intensity: 100 µW/cm^2	104, 103	

Bacteria

Aeromonas hydrophila

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^4-10^5 cfu/ml	UV	3,2 sec	3,3 – 5,3 mWs/cm^2		12,5	> 99.0% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,0 – 4,75 mWs/cm^2		12,5	> 99.3% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,5 mWs/cm^2		12,5	> 99.8% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
10^3 cfu	UV		5 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	
10^4-10^5 cfu/ml	UV	3,2 sec	12 - 30 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	13 - 29 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	21 - 24 mWs/cm^2		12,5	> 99.9% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
1,3 x10^7 $TCID_{50}$/ml	UV		23,1 mWs/cm^2			> 4 log reduction		60	

Conclusion: *A. hydrophila* is susceptible to UV irradiation with 3 log reduction when exposed to 5-25 mWs/cm^2.

Aeromonas punctata

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^3 cfu	UV		4 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	
2,2 x10^5 $TCID_{50}$/ml	UV		23,1 mWs/cm^2			99,97% reduction		60	

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	UV		0,05 mW/cm^2	7,2	7	99,9% reduction	PBS	71	Reduction rate: 0,15/sec
10^7 CFU/ml	UV		0,05 mW/cm^2	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,14/sec

10^7 CFU/ml	UV	48 sec	2,34 mWs/cm^2	7,2	7	99,9 % reduction	PBS	71	
10^7 CFU/ml	UV	50 sec	2,38 mWs/cm^2	7,8	7	99,9 % reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	
10^4-10^5 cfu/ml	UV	3,2 sec	3,3 – 5,3 mWs/cm^2		12,5	> 99.0% reduction	Water with dissolved organic matter without filtration.	15	
	UV		3,4 mWs/cm^2			3 log reduction		86	
10^3 cfu	UV		4 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	
10^4-10^5 cfu/ml	UV	3,2 sec	4,0 – 4,75 mWs/cm^2		12,5	> 99.3% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,5 mWs/cm^2		12,5	> 99.8% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	12 - 30 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	13 - 29 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter with filtration.	15	
	UV		13 mWs/cm^2		12,5	”inactivated”	Infection trial in water filtratrated with 25 nm filter followed by UV irradiation.	15	98,5% mortality in control group.
10^4-10^5 cfu/ml	UV	3,2 sec	21 - 24 mWs/cm^2		12,5	> 99.9% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
5,6 x10^6 $TCID_{50}$/ml	UV		23,1 mWs/cm^2		18,3	> 4 log reduction		60	

Conclusion: *A. salmonicida* is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 5-25 mWs/cm^2.

Escherichia coli

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^3 cfu	UV		4 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	

Natural flora (heterothrophic bacteria)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{3,5}$ cfu/ml	UV					93,6% reduction	flowrate 4 l/m	53	Sea water, filtrated (10 µm)
7,4 x 10^5 cfu/ml	UV		10 mWs/cm^2		5	2,5 log reduction	Sea water, Artemia added.	69	
7,4 x 10^5 cfu/ml	UV		13 mWs/cm^2		5	2,5 log reduction	Sea water, Artemia added.	69	The artemia protects the bacteria.
7,4 x 10^5 cfu/ml	UV		22 mWs/cm^2		5	2,5 log reduction	Sea water, Artemia added.	69	The artemia protects the bacteria.
7,4 x 10^5 cfu/ml	UV med præfiltrering		22 mWS/cm^2		5	> 5 log reduction	Sea water, Artemia added, filtration through 50 µm	69	cfu after filtration app. The same as before filtration.
4,7 x 10^4 cfu/ml	UV		150 mWs/cm^2			4 log reduction	flowrate 2,0 m^3	56	Natural flora in wastewater from hathing facility.
app. 9000 cfu/ml	UV		app. 1800 mWs/cm^2	7,5		1,7 log reduction	Fish farm, recirculation.	88	

Conclusion: there are conflicting results concerning the resistance of the natural flora towards UV irradiation. The composition of the natural flora will depend on a lot of different variables, which will influence the effect of UV irradiation.

Pseudomonas flourescens

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^4-10^5 cfu/ml	UV	3,2 sec	3,3 – 5,3 mWs/cm^2		12,5	> 99.0% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,0 – 4,75 mWs/cm^2		12,5	> 99.3% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,5 mWs/cm^2		12,5	> 99.8% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
10^3 cfu	UV		5 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	
10^4-10^5 cfu/ml	UV	3,2 sec	12 - 30 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	13 - 29 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	21 - 24 mWs/cm^2		12,5	> 99.9% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
1,5 x10^7 $TCID_{50}$/ml	UV		23,1 mWs/cm^2		20,4	4 log reduction		60	

Conclusion: *P. flourescens* is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 5-25 mWs/cm^2.

Vibrio anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		2,9 mWs/cm^2			3 log reduction		86	
10^4-10^5 cfu/ml	UV	3,2 sec	3,3 – 5,3 mWs/cm^2		12,5	> 99.0% reduction	Water with dissolved organic matter without filtration.	15	
10^3 cfu	UV		4 mWs/cm^2			≥ 99,9% reduction	Spreadning on agar plate followed by UV irradiation.	104, 59	
10^4-10^5 cfu/ml	UV	3,2 sec	4,0 – 4,75 mWs/cm^2		12,5	> 99.3% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4,5 mWs/cm^2		12,5	> 99.8% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	12 – 30 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	13 - 29 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	21 – 24 mWs/cm^2		12,5	> 99.9% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
1,9 x10^6 $TCID_{50}$/ml	UV		23,1 mWs/cm^2		20,3	> 5 log reduction		60	

Conclusion: *V. anguillarum* is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 5-25 mWs/cm^2.

Vibrio ordalii

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		5,5 mWs/cm^2			3 log reduction		86	

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^4-10^5 cfu/ml	UV	3,2 sec	3,3 – 5,3 mWs/cm^2		12,5	> 99.0% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	4 – 4,75 mWs/cm^2		12,5	> 99.3% reduction	Water with dissolved organic matter with filtration.	15	

10^4-10^5 cfu/ml	UV	3,2 sec	4,5 mWs/cm^2		12,5	> 99.8% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	12 - 30 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter without filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	13 – 29 mWs/cm^2		12,5	> 99.9% reduction	Water with dissolved organic matter with filtration.	15	
10^4-10^5 cfu/ml	UV	3,2 sec	21 – 24 mWs/cm^2		12,5	> 99.9% reduction	Spring water, 25 nm filtration before UV irradiation.	15	
$10^{5,21}$ cfu/ml	UV, after ferrichlorid precipitation		250 mWs/cm^2		65	1 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
	UV		1200 mWs/cm^2			2½ log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	forfilter 20 µm

Conclusion: In laboratory trials *Y. ruckeri* is susceptible to UV irradiation with ≥ 3 log reduction when exposed to 5-25 mWs/cm^2. In full scale trials, after precipitation using ferrichlorid only 1 log reduction was obtained after a dose of 250 mWs/cm^2, and it was not possible to obtain a 3 log reduction using a dose of 1200 mWs/cm^2 despite prefiltration through a 20 µm filter.

Parasites

Ichthyophthirius multifiliis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		92 mWs/cm^2	6,1-7,3		Transmission prevented	Transmission of Ich from infected to free fish.	34	1 UV lamp.

Myxosoma cerebralis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		4 mWs/cm^2			> 5 log reduction	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	UV, pre filtration of water		28 mWs/cm^2			86-100% reduction af infektivitet	25 µm filter, UV irradiation of contaminated water, fish added, clinic and % spores registered.	41	Ingen klinik, ingen spores i det ene forsøg, 14% spores i det andet forsøg. Kontrolfisk 100% spores og klinik.

	UV		35 mWs/cm^2			”inactivated”	Infection trial wiht UV irradiated spores (UV-doses 35000, 43000 and 112000 µWs/cm2)	40	
2 x 10^4 TAM	UV		40 mWS/cm^2			”inactivated”	Smitteforsøg med UV-behandlede triactinomyxoner (1000/fisk)	36	
	UV		48 mWs/cm^2			4,75 log reduction	Infektivitet af myxospores i tubifex	37	Myxospore suspension
	UV		1700 mWs/cm^2			alle døde	myxospores i suspension	44	

Fungae and oomycetes

Achlya flagellata

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		220 mWs/cm^2			”inactivated”		59	

Aphanomyces laevis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV		210 mWs/cm^2			”inactivated”		59	

Saprolegnia

Pathogen	Concentration pathogen	Disin-fectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
Saprolegnia anispora		UV		150 mWs/cm^2			”inactivated”	Punched agar disk with hyphae irradiated.	104, 59	
Saprolegnia parasitica		UV		200 – 230 mWs/cm^2			”inactivated”	Punched agar disk with hyphae irradiated.	104, 59	
Saproglegnia sp.		UV		210 – 250 mWs/cm^2			”inactivated”	Punched agar disk with hyphae irradiated.	104, 59	

UV in combination with other treatments

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	UV/ozone		161 mWs/cm^2 + ozone			Survival		86	

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	UV/NaOCl		0,05 mW/cm^2 / 0,2 mg/l added	7,2	7	99,9% reduction	PBS	71	Reduction rate: 0,32/sec
10^7 CFU/ml	UV/NaOCl		0,05 mW/cm^2 / 2,0 mg/l added	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,26/sec
10^7 CFU/ml	UV/I_2		0,05 mW/cm^2 / 1,0 mg/l added	7,2	7	99,9 % reduction	PBS	71	Reduction rate: 0,42/sec
10^7 CFU/ml	UV/I_2		0,05 mW/cm^2 / 1,3 mg/l added	7,8	7	99,9 % reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,28/sec
10^7 CFU/ml	UV/ozone		0,05 mW/cm^2 / 0,1 mg/l	7,2	7	99,9 % reduction	PBS	71	Reduction rate: 0,32/sec

Natural flora (heterothrophic bacteria)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
116 ± 25 cfu/ml	ozone/UV		0,21 mg/l, 54,7 mJ/cm^2	7,5	14,3	1,81 log reduction	fish farm, recirculation	87	

Miscellaneous chlorine compounds

NB: It it not always stated if the concentration of the disinfectant is as free chlorine or as the disinfectant itself.

Virus

VHSV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{7,8}$ $TCID_{50}$/ml	Benzalkonium chlorid	6 hours	1%			Stable	10 % calf serum added	4, 5	
10^{8} $TCID_{50}$/ml	Benzalkonium chlorid	30 min	1:1000		15	Not detectable	10% (w/v) dilution in PBS. 1% fetal calf serum.	65	Strain JF00Ehi1. Benzalkoniumklorid 10% (w/v). Dilution scale 1:1000.
10^{8} $TCID_{50}$/ml	Benzalkonium chlorid	5 min	1:1000		15	Not detectable	10% (w/v) dilution in artificail sea water. 1% fetal calf serum.	65	Strain JF00Ehi1. Benzalkoniumklorid 10% (w/v). Dilution scale 1:1000.
$10^{7,2}$ $TCID_{50}$/ml	NaOCl	10 min	7,6 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,8}$ $TCID_{50}$/ml	NaOCl	60 min	7,6 mg/ml Cl_2	7,07-7,49	10	Stable	2,5 % calf serum added	4	
$10^{6,2}$ $TCID_{50}$/ml	NaOCl	5 min	25 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	10 min	25 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	2 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	10 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	5 min	54 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,8}$ $TCID_{50}$/ml	NaOCl	5 min	54 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{7,8}$ $TCID_{50}$/ml	NaOCl	2 min	98 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{7,2}$ $TCID_{50}$/ml	NaOCl	10 min	98 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	< 2 min	515 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,8}$ $TCID_{50}$/ml	NaOCl	< 2 min	515 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
10^{7} $TCIID_{50}$/ml	NaOCl	1 min	50 ppm		15	Not detectable	Diluted in PBS. 1% fetal calf serum.	65	Isolat JF00EHi1. Dilution scale 1:50.
10^{7} $TCID_{50}$/ml	NaOCl	1 min	50 ppm		15	Ineffective.	Diluted in artifical sea water. 1% fetal calf serum.	65	Isolat JF00EHi1. Dilution scale 1:50.
10^{7} $TCID_{50}$/ml	NaOCl	5 min	100 ppm		15	Not detectable	Diluted in artifical sea water. 1% fetal calf serum.	65	Isolat JF00EHi1. Dilution scale 1:50
10^{7} $TCID_{50}$/ml	NaOCl	1 min	200 ppm		15	Not detectable	Diluted in artifical sea water. 1% fetal calf serum.	65	Isolat JF00EHi1. Dilution scale 1:50

Conclusion: In laboratory experiements under dirty conditions (addition of 1% calf serum) it was possible to decimate VHSV to non detectable by use of 100 mg/l chlorine for 5 min. Another experiment showed that using the same dose it takes 2 min to decimate VHSV ≥ 2 log under clean conditions but 10 min under dirty conditions (2,5% calf serum). It is not possible to validate the dose to decimate VHSV 3 log based on these figures.

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^4 - $10^5$$TCID_{50}$/ml	NaOCl	30 sec	0,1 mg/l residual	6,9	10	”inactivated”	Distilled water.	99, 98	
10^4 - $10^5$$TCID_{50}$/ml	NaOCl	5 min	0,5 mg/l residual	6,9	10	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$	99, 98	
10^4 - $10^5$$TCID_{50}$/ml	NaOCl	10 min	0,5 mg/l residual	8,2	10	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$	99, 98	
10^4 - $10^5$$TCID_{50}$/ml	NaOCl	30 sec	1,0 mg/l residual	8,2	10	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$	99, 98	
	NaOCl	30 min	10 ppm Cl_2			”inactivated”		8	

Conclusion: Chlorine is effective to inactive IHNV at low cencentrations of short contact time. The tests are performed under fairly clean conditions and not under conditions comparable to wastewater from fish cutting plants.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
6,8 log10 ffu/ml	chloramine-T	5 min	0,25%		4	> 6,5 log reduction	Hard water, 342 ppm total hardness.	89	Buffodine
6,2 log10 ffu/ml	ClO_2	5 min	25 ppm		4	1,2 log reduction	Hard water, 342 ppm total hardness, no addition of serum.	89	Zydox AD-05
5,1 log10 ffu/ml	ClO_2	5 min	50 ppm		4	1,4 log reduction	Hard water, 342 ppm total hardness, addition of serum.	89	Zydox AD-05
5,1 log10 ffu/ml	ClO_2	5 min	50 ppm		4	> 4,8 log reduction	Hard water, 342 ppm total hardness, addition of serum.	89	Zydox AD-05
6,2 log10 ffu/ml	ClO_2	5 min	50 ppm		4	5,3 log reduction	Hard water, 342 ppm total hardness, no addition of serum.	89	Zydox AD-05
5,5 log10 ffu/ml	OCl^-	5 min	100 ppm		4	> 5,2 log reduction	Hard water, 342 ppm total hardness, with and without addition of serum.	89	

	NaOCl	15 min	100 mg/l	-	-	"inactivated"	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Testet ved konc. 5, 10, 20, 50 og 100 mg/l med kontakttid 15 min og 30 min.

Conclusion: Under laboratory conditions it is possible to obtain 5 log reduction of ISAV using 100 mg/l chlorine for 5 minuts.

KHV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 - 1,5 x 10^4 $TCID_{50}$/ml	Benzalkonium chloride	30 sec	60 mg/l		0	Not detectable	Virus and disinfectant mixed 1:1, tested after 30 sek and 20 min. Diluted 1:10 using L15 medium og inoculated 200 µl.	55	Strain KHV-I. The method cannot detect a 3 log reduction.
1 - 1,5 x 10^4 $TCID_{50}$/ml	Benzalkonium chloride	30 sec	30 mg/l		25	Not detectable	Virus and disinfectant mixed 1:1, tested after 30 sek and 20 min. Diluted 1:10 using L15 medium og inoculated 200 µl.	55	Strain KHV-I. The method cannot detect a 3 log reduction.
	NaOCl	20 min	0,3 mg/l residual			98,5% reduction		55	Strain KHV-I.
1 - 1,5 x 10^4 $TCID_{50}$/ml	NaOCl	20 min	200 mg/l		0	"inactivated"	Virus and disinfectant mixed 1:1, tested after 30 sec and 20 min. Diluted 1:10 using L15 medium og inoculated 200 µl.	99, 98	Strain KHV-I. The method cannot detect a 3 log reduction.
1 - 1,5 x 10^4 $TCID_{50}$/ml	NaOCl	20 min	250 mg/l		25	"inactivated"	Virus and disinfectant mixed 1:1, tested after 30 sec and 20 min. Diluted 1:10 using L15 medium og inoculated 200 µl.	99, 98	Strain KHV-I. The method cannot detect a 3 log reduction.

Conclusion: It does seem as if KHV is susceptible to chlorine. It is difficult to assess the dose to reduce the titer 3 log for KHV, as the methods used are not able to detect such a reduction.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{7,5}$ $TCID_{50}$/ml	Benzalkonium chlorid	6 hours	1%			Stable	10 % calf serum added	4, 5	Trade name Mefarol.
	chloramine-T	30 min	3,2%		4	> 4 log reduction	Clean conditions.	49	

$10^{5,5}$ $TCID_{50}$/ml	chloramine-T (SETAX)	24 hours	50 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{5,5}$ $TCID_{50}$/ml	chloramine-T (SETAX)	24 hours	100 mg/l	7,5		2 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{5,5}$ $TCID_{50}$/ml	chloramine-T (SETAX)	1 hour	250 mg/l	7,5		4 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
10^{6} infectious units/ml	chloramine-T (SETAX)	30 min	3 g/l		4	1 log reduction	Sea water added 10% salmon blood.	22	
10^{6} infectious units/ml	chloramine-T (SETAX)	30 min	10 g/l		4	2 log reduction	Sea water added 10% salmon blood.	22	
10^{5} $TCID_{50}$/ml	Cl_2	30 min	25 ppm (=25 mg/l)		room temp.	”inactivated”	Tap water.	17	Chlorine concentration after correction for medium and diluent addition.
10^{5} $TCID_{50}$/ml	Cl_2	30 min	25 ppm		room temp.	”inactivated”	PBS	17	Chlorine concentration after correction for medium and diluent addition.
$10^{7,5}$ $TCID_{50}$/ml	Cl_2	30 min	40 ppm		room temp.	”inactivated”	Salt water.	17	Chlorine concentration after correction for medium and diluent addition.
10^{5} $TCID_{50}$/ml	NaOCl	1 min	0,1 mg/l clorine residual	6,9	10	”inactivated”	Distilled water.	99	
10^{5} $TCID_{50}$/ml	NaOCl	10 min	0,2 mg/l clorine residual	8,2	10	Stable	Hard lake water.	99	
10^{5} $TCID_{50}$/ml	NaOCl	2 min	0,7 mg/l clorine residual	8,2	10	”inactivated”	Hard lake water.	99	
10^{5} $TCID_{50}$/ml	NaOCl	10 min	0,2 mg/l clorine residual	8,2	10	”inactivated”	Soft lake water.	99	
$10^{3,8}$ $TCID_{50}$/ml	NaOCl	60 min	7,5 mg/ml Cl_2	7,07-7,49	10	Stable		4	
$10^{4,2}$ $TCID_{50}$/ml	NaOCl	60 min	7,5 mg/ml Cl_2	7,07-7,49	10	Stable	2,5 % calf serum added	4	
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	2 min	30 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	

$10^{6,2}$ $TCID_{50}$/ml	NaOCl	30 min	30 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{6,2}$ $TCID_{50}$/ml	NaOCl	2 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,2}$ $TCID_{50}$/ml	NaOCl	20 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	2 min	56 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{5,2}$ $TCID_{50}$/ml	NaOCl	20 min	56 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{6,5}$ $TCID_{50}$/ml	NaOCl	2 min	106 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{6,2}$ $TCID_{50}$/ml	NaOCl	20 min	106 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
$10^{5,2}$ $TCID_{50}$/ml	NaOCl	< 2 min	520 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	< 2 min	520 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
10^{4} - 10^{5} $TCID_{50}$/ml	NaOCl	60 sec	0,1 mg/l residual	6,9	10	”inactivated”	Distilled water.	98	
10^{4} - 10^{5} $TCID_{50}$/ml	NaOCl	10 min	0,2 mg/l residual	6,9	10	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$	98	
10^{4} - 10^{5} $TCID_{50}$/ml	NaOCl	2 min	0,7 mg/l residual	8,2	10	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$	98	
10^{4} - 10^{5} $TCID_{50}$/ml	NaOCl	10 min	0,2 mg/l residual	8,2	10	Stable	Hard lake water., 120 mg/l $CaCO_3$	98	
$10^{3,9}$ $TCID_{50}$/ml	NaOCl	5 min	1 mg/l clorine residual		21	”inactivated”	Tested using 0,13, 0,25, 0,5, 1, 2, 4, 8 and 16 mg/l residual. Distilled water..	23	IPNV: Serotype Buhl.
$10^{4,5}$ $TCID_{50}$/ml	NaOCl	5 min	4 mg/l clorine residual	6,6-8,9	21	”inactivated”	Tested using 0,13, 0,25, 0,5, 1, 2, 4, 8 and 16 mg/l residual. Distilled water..	23	IPNV: Serotype Buhl.
$10^{4,3}$ $TCID_{50}$/ml	NaOCl	15 sec	5 mg/l clorine residual		21	”inactivated”	Ttested at time 0, 15, 30, 60, 120 s. Distilled water.	23	IPNV: Serotype Buhl.
$10^{4,5}$ $TCID_{50}$/ml	NaOCl	5 min	16 mg/l clorine residual	9,0-10,0	21	”inactivated”	Tested using 0,25, 0,5, 1, 2, 4, 8 and 16 mg/l residual. Distilled water.	23	IPNV: Serotype Buhl.
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	24 hours	50 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	24 hours	100 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{5,5}$ $TCID_{50}$/ml	NaOCl	1 hour	250 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.

$10^{6,6}$ $TCID_{50}$/ml	NaOCl	10 hours	43 mg/l		7	1 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{6,6}$ $TCID_{50}$/ml	NaOCl	10 hours	130 mg/l		7	5 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{6,6}$ $TCID_{50}$/ml	NaOCl	10 hours	260 mg/l		7	4 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{6,6}$ $TCID_{50}$/ml	NaOCl	5 hours	130 mg/l		7	3 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{6,6}$ $TCID_{50}$/ml	NaOCl	5 hours	260 mg/l		7	3½ log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	

Conclusion: In laboratory experiments under highly contaminated conditions that exist in process and wastewater from fish slaughterhouses chlorine in a dose of 130 mg/l with a contact time of 10 hours gave 5 log reduction in titer. During full-scale trials it has not been possible to achieve these results. It was, for example, not possible in a full-scale test to inactivate IPNV using NaOCl at a concentration of 250 mg/l chlorine and a contact time of 1 hour. Using Chloramine-T has it been possible with a contact time of 1 hour and concentration of 250 mg/l chlorine to achieve a 4 log inactivation in a full-scale trials. In the full-scale trials no continous stirring was performed and pockets of non /low treated areas may have occurred and/or the virus may have been protected by different aggregations.

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10 µg purified virus	Benzalkonium chlorid	10 min	50 µg/ml		20	”Effective”	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae. Concentration testet: 2,5 - 100 µg/ml.	9	Isolate: SJNNV
10 µg purified virus	CaOCl	10 min	50 µg/ml		20	”Effective”	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae. Concentration testet: 2,5 - 100 µg/ml.	9	Isolate: SJNNV

$10^{7,25\ TCID}{}_{50}$/ml	Cl_2	5 min	50 ppm		15	Not detectable	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{7,25\ TCID}{}_{50}$/ml	Cl_2	30 min	25 ppm		15	Not detectable	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{7,25\ TCID}{}_{50}$/ml	Cl_2	30 min	100 ppm		15	2 log reduction	HBSS + calf serum. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
10 µg purified virus	NaOCl	10 min	50 µg/ml		20	”Effective”	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae. Concentration testet: 2,5 - 100 µg/ml.	9	Isolate: SJNNV

Conclusion: Under laboratory settings and clean conditions nodavirus is easily incativated using a dose of 50 mg/l for 5 minuts. When adding calf serum it was only possible to obtain a 2 log reduction using a concentration of 100 mg/l for 30 minuts.

Hirame rhabdovirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	OCl^-	1 min	0,42 mg/l			>99% reduction		57	Hypoclorite produced by use of batch electrolytic system.
$10^{4,5\ TCID}{}_{50}$/ml	OCl^-	1 min	0,34 mg/l			3 log reduction	flowrate 3,5 m^3/t, el. 1,5 A	54	Electrolyzed salt water.
$10^{4,5\ TCID}{}_{50}$/ml	OCl^-	2,5 min	0,49 mg/l			> 4 log reduction	flowrate 3,5 m^3/t, el. 2 A	54	Electrolyzed salt water.

Conclusion: Electrolysation of saltwater to produce chlorine seem to be an effective method to inactivate hirame rhabovirus using a dose of 0,5 mg/l and a contact time of 2,5 minuts. There has not been tested under dirty conditions, which may reduce the effect of chlorine.

oncorhynchus masou virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Benzalkonium chlorid	30 sec	100 ppm		0	”inactivated”		35	
	Benzalkonium chlorid	30 sec	100 ppm		25	”inactivated”		35	
	Benzalkonium chlorid	20 min	100 ppm		0	”inactivated”		35	
	Benzalkonium chlorid	20 min	100 ppm		15	”inactivated”		35	

	NaOCl	30 sec	100 ppm		0	”inactivated”		35	
	NaOCl	30 sec	100 ppm		25	”inactivated”		35	
	NaOCl	20 min	50 ppm		0	”inactivated”		35	
	NaOCl	20 min	50 ppm		15	”inactivated”		35	

Conclusion: Under laboratory conditions *O. masou* virus is inactivated using a dose of 100 mg/l for 30 sec, or 50 mg/l for 20 min.

PFRV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
6,2 log10 $TCID_{50}$/ml	Benzalkonium chlorid	6 hours	1%			Stable	10 % calf serum added	4	Trade name Mefarol.
6,5 log10 $TCID_{50}$/ml	NaOCl	20 min	7,6 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
5,8 log10 $TCID_{50}$/ml	NaOCl	60 min	7,6 mg/ml Cl_2	7,07-7,49	10	Stable	2,5 % calf serum added	4	
6,5 log10 $TCID_{50}$/ml	NaOCl	20 min	25 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,2 log10 $TCID_{50}$/ml	NaOCl	20 min	25 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
6,8 log10 $TCID_{50}$/ml	NaOCl	20 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,8 log10 $TCID_{50}$/ml	NaOCl	20 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
6,8 log10 $TCID_{50}$/ml	NaOCl	5 min	54 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,5 log10 $TCID_{50}$/ml	NaOCl	10 min	54 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
6,8 log10 $TCID_{50}$/ml	NaOCl	5 min	101 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
7,2 log10 $TCID_{50}$/ml	NaOCl	10 min	101 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
6,5 log10 $TCID_{50}$/ml	NaOCl	< 2 min	540 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,5 log10 $TCID_{50}$/ml	NaOCl	2 min	540 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	

Conclusion: As the author has not noted the precise reduction it is difficult to evalute which dose to use to inactivate PFRV. It does seem, though, as PFRV is susceptible to chlorine when using the right dose for the right time.

Ranavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 x 10^7 PFU/ml	Chlorhexidine	1 min	0,005%		22	1,7 log reduction	Novalsan 0,25%	14	Isolate from American bullfrog
1 x 10^7 PFU/ml	Chlorhexidine	5 min	0,005%		22	2,6 log reduction	Novalsan 0,25%	14	Isolate from American bullfrog
1 x 10^7 PFU/ml	Chlorhexidine	1 min	0,015%		22	3,25 log reduction	Novalsan 0,75%	14	Isolate from American bullfrog
1 x 10^7 PFU/ml	Chlorhexidine	1 min	0,040%		22	3,75 log reduction	Novalsan 2,0%	14	Isolate from American bullfrog
1 x 10^7 PFU/ml	NaOCl	1 min	0,012%		22	0,5 log reduction		14	Isolate from American bullfrog
1 x 10^7 PFU/ml	NaOCl	5 min	0,012%		22	0,5 log reduction		14	Isolate from American bullfrog
1 x 10^7 PFU/ml	NaOCl	1 min	0,060%		22	0,9 log reduction		14	Isolate from American bullfrog
1 x 10^7 PFU/ml	NaOCl	5 min	0,060%		22	1,8 log reduction		14	Isolate from American bullfrog
1 x 10^7 PFU/ml	NaOCl	1 min	0,18% (1,8 g/l)		22	”inactivated”		14	Isolate from American bullfrog
	NaOCl	5 hours	400 mg/l			Survival	Virus i udtørret cellekulturmedium overhældt med NaOCl. Testet efter 2 og 5 hours.	67	ENV
	NaOCl	2 hours	200 mg/l			”inactivated”	Virus in cell culture medium.. Kun testet efter 2 hours og ved denne dosis.	67	EHNV

Conclusion: It seems as if ranavirus is quite resistant to chlorine.

SVCV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
6,5 log10 $TCID_{50}$/ml	Benzalkonium chlorid	6 hours	1%			Stable	10 % calf serum added	4, 5	
$10^{7,1}$ $TCID_{50}$/ml	Benzalkonium chlorid	20 min	100 ppm		22	> 4 log reduction	Diluted in PBS. Contact time 30 sec or 20 min. 1%	61	Isolate S30

							calf serum.		
6,2 log10 $TCID_{50}$/ml	NaOCl	20 min	7,6 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,8 log10 $TCID_{50}$/ml	NaOCl	60 min	7,6 mg/ml Cl_2	7,07-7,49	10	Stable	2,5 % calf serum added	4	
6,5 log10 $TCID_{50}$/ml	NaOCl	10 min	27 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,5 log10 $TCID_{50}$/ml	NaOCl	10 min	27 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
7,5 log10 $TCID_{50}$/ml	NaOCl	2 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
7,2 log10 $TCID_{50}$/ml	NaOCl	10 min	36 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
5,8 log10 $TCID_{50}$/ml	NaOCl	2 min	55 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
5,5 log10 $TCID_{50}$/ml	NaOCl	5 min	55 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
5,5 log10 $TCID_{50}$/ml	NaOCl	< 2 min	101 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
5,8 log10 $TCID_{50}$/ml	NaOCl	2 min	101 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	
6,2 log10 $TCID_{50}$/ml	NaOCl	< 2 min	506 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction		4	
6,5 log10 $TCID_{50}$/ml	NaOCl	2 min	506 mg/ml Cl_2	7,07-7,49	10	≥ 99 % reduction	2,5 % calf serum added	4	

Conclusion: As the author has not noted the precise reduction it is difficult to evalute which dose to use to inactivate PFRV. It does seem, though, as PFRV is susceptible to chlorine when using the right dose for the right time.

Yellowtail ascites virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	OCl^-	1 min	0,42 mg/l			>99% reduction		57	Hypoclorite produced by use of batch electrolytic system.
$10^{4,5}$ $TCID_{50}$/ml	OCl^-	1 min	0,58 mg/l			> 4 logi naktivering	flowrate 3,5 m^3/t, el. 2.5 A	54	Electrolyzed salt water.
$10^{4,5}$ $TCID_{50}$/ml	OCl^-	1 min	0,45 mg/l			3 log reduction	flowrate 3,5 m^3/t, el. 2 A	97	Electrolyzed salt water.

Bacteria

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		5	Stable	Test of effect of Temperature.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		15	3 log reduction	Test of effect of Temperature.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		25	> 4½ log reduction	Test of effect of Temperature.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		15	2 log reduction	Artificial sea water.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		15	3 log reduction	Hard water (300 ppm $CaCO_3$)	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	3 min	0,02%		15	4½ log reduction	Distilled water.	85	
10^{5} cfu/ml	Benzalkonium chlorid	5 min	0,03%		20	> 4 log reduction		85	Concentration of commercial product.
10^{8} cfu/ml	Benzalkonium chlorid	4 min	0,03%		20	> 7 log reduction	Test of effect of bacteria titer.	85	
$10^{6,5}$ cfu/ml	Benzalkonium chlorid	2 min	0,03%		20	> 5½ log reduction	Test of effect of bacteria titer.	85	
10^{5} cfu/ml	Benzalkonium chlorid	1 min	0,03%		20	> 4 log reduction	Test of effect of bacteria titer.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	1 min	0,03%		20	3½ log reduction	300 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	Benzalkonium chlorid	1 min	0,03%		20	4½ log reduction	0 ppm calf serum added.	85	
10^{5} cfu/ml	Benzalkonium chlorid	1 min	0,1%		20	> 4 log reduction		85	Concentration of commercial product.
	chloramine-T	30 min	0,08 - 0,5 % (v/v)		4	> 5 log reduction	Tested at 0,001, 0,01, 0,05, 0,08, 0,1, 0,5, 0,7 and 1%. Hard water, organic loaded.	49	
10^{8}-10^{9} CFU/ml	chloramine-T (SETAX)	1 min	1 g/l		4	≥ 6 log reduction	Sea water added 10% salmon blood.	22	
10^{3} cells/ml	NaOCl	10 min	0,01 mg/l residual	6,9	20	Stable	Distilled water.	97, 98	Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.
10^{3} cells/ml	NaOCl	1 min	0,01 mg/l residual	6,9	20	2 log reduction	Distilled water.	97, 98	Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.
10^{3} cells/ml	NaOCl	10 min	0,05 mg/l residual	6,9	20	Stable	Soft lake water., 30 mg/l $CaCO_3$	97, 98	

10^3 cells/ml	NaOCl	10 min	0,05 mg/l residual	8,2	20	Stable	Hard lake water., 120 mg/l $CaCO_3$	97, 98	
10^3 cells/ml	NaOCl	30 sec	0,1 mg/l residual	6,9	20	”inactivated”	Distilled water.	97, 98	Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.
10^3 cells/ml	NaOCl	30 sec	0,1 mg/l residual	6,9	20	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$	97, 98	
10^3 cells/ml	NaOCl	30 sec	0,2 mg/l residual	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$	97, 98	
10^7 CFU/ml	NaOCl	1 min	0,2 mg/l added	7,2	7	4 log reduction	PBS	71	Read off a graph.
10^7 CFU/ml	NaOCl	36 sec	0,2 mg/l added	7,2	7	99,9 % reduction	PBS	71	
10^7 CFU/ml	NaOCl		0,2 mg/l added	7,2	7	99,9% reduction	PBS	71	Reduction rate: 0,20/sec
10^7 CFU/ml	NaOCl	1 min	2 mg/l added	7,8	7	4 log reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Read off a graph.
10^7 CFU/ml	NaOCl		2,0 mg/l added	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,19/sec
10^7 CFU/ml	NaOCl		4,0 mg/l added	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,30/sec
$10^{5,5}$ cfu/ml	NaOCl	5 min	4 ppm		15	> 4½ log reduction	Artificial sea water.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	4 ppm		15	4½ log reduction	Hard water (300 ppm $CaCO_3$)	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	4 ppm		15	> 4½ log reduction	Distilled water.	85	
10^8 cfu/ml	NaOCl	5 min	5 ppm		20	Stable	Test of effect of bacteria titer.	85	Concentration of commercial product. (Purelox)
10^6 cfu/ml	NaOCl	5 min	5 ppm		20	> 5 log reduction	Test of effect of bacteria titer.	85	Concentration of commercial product. (Purelox)
10^4 cfu/ml	NaOCl	3 min	5 ppm		20	> 3 log reduction	Test of effect of bacteria titer.	85	Concentration of commercial product. (Purelox)
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		20	Stable	300 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		20	3 log reduction	10 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		20	4½ log reduction	0 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		5	4 log reduction	Test of effect of Temperature.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		15	4½ log reduction	Test of effect of Temperature.	85	
$10^{5,5}$ cfu/ml	NaOCl	1 min	5 ppm		25	> 4½ log reduction	Test of effect of Temperature.	85	

10^5 cfu/ml	NaOCl	1 min	10 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Purelox)
3,8 x 10^6 cfu/ml	OCl^-	1 min	0,11 mg/l			4 log reduction	flowrate 3,0 m^3/t, el. 0,5 A	54	Electrolyzed salt water.
3,8 x 10^6 cfu/ml	OCl^-	1 min	0.06 mg/l			3 log reduction	flowrate 3,5 m^3/t, el. 0,5 A	54	Electrolyzed salt water.

Conclusion: Under laboratory condtions *Aeromonas salmonicida* is sensitive to chlorine.

Carnobacterium piscicola

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	chloramine-T	30 min	0,1 - 0,5 % (v/v)		4	> 5 log reduction	Tested at 0,001, 0,01, 0,05, 0,08, 0,1, 0,5, 0,7 and 1%. Hard water, organic loaded.	49	

Edwardsiella tarda

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	NaOCl	20 min	400 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	NaOCl	60 min	400 ppm		20	5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	20 min	3200 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary

							hours.		concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	60 min	3200 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	20 min	400 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	60 min	200 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Conclusion: According to this paper *E. tarda* is quite resistant to chlorine!

Indigenious flora (heterothophic bacteria)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	OCl^-	1 min	0,54 mg/l			>99% reduction	Hypoclorite produced by use of batch electrolytic system.	57	hatchery inlet water
	OCl^-	1 min	0,64 mg/l			>99% reduction	Hypoclorite produced by use of batch electrolytic system.	57	waste-seawater
$4,7 \times 10^4$ cfu/ml	OCl^-	1 min	1,28 mg/l			3 log reduction	flowrate 2,0 m^3/t, el. 2,5 A (Electrolyzed salt water)	56	Natural flora in wastewater from hathing

									facility.
$10^{3,5}$ cfu/ml	OCl^-			8,2		Stable	flowrate 4 l/min, el. 0,1 A (Electrolyzed salt water)	53	Sea water, filtrated (10 µm).
$10^{3,5}$ cfu/ml	OCl^-			8,2		99,4% reduction	flowrate 4 l/min, el. 1,2 A. (Electrolyzed salt water)	53	Sea water, filtrated (10 µm).
$10^{3,5}$ cfu/ml	OCl^-		2,13 mg Cl/l	8,2		”inactivated”	flowrate 4 l/min, el. 1,3 A. (Electrolyzed salt water)	53	Sea water, filtrated (10 µm).

Conclustion: When electrolysing saltwater it seems possible to obtain a 3 log reduction of the natural flora. Wether this will also be the case in wastewater from fish cutting plants is unknown.

Lactococcus garviae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	chloramine-T	30 min	0,08 - 0,5 % (v/v)		4	> 5 log reduction	Tested at 0,001, 0,01, 0,05, 0,08, 0,1, 0,5, 0,7 and 1%. Hard water, organic loaded.	49	

Renibacterium salmoninarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
3×10^4 - 2×10^6 bacteria/ml	NaOCl	26 sec	0,05 mg/l free chlorine	7	15	3 log reduction	in vitro in PBS	82	Read off a graph.
3×10^4 - 2×10^6 bacteria/ml	NaOCl	42 sec	0,6 mg/l free chlorine	7	15	5 log reduction	in vitro in PBS	82	Read off a graph.
5×10^4 bacteria/ml	NaOCl	20 sec	0,06 mg/l free chlorine	7	15	3 log reduction	in vitro in PBS	82	Read off a graph.
5×10^4 bacteria/ml	NaOCl	120 sec	0,07 mg/l free chlorine	7	7,5	3 log reduction	in vitro in PBS	82	Read off a graph.
5×10^4 bacteria/ml	NaOCl	54 sec	0,41 - 0,53 mg/l free chlorine	6	15	3 log reduction	in vitro in PBS	82	Read off a graph.
5×10^4 bacteria/ml	NaOCl	≥ 60 sec	0,41 - 0,53 mg/l free chlorine	7	15	3 log reduction	in vitro in PBS	82	Read off a graph., at t=120 sec 2,7 log reduction
5×10^4 bacteria/ml	NaOCl	92 sec	0,41 - 0,53 mg/l free chlorine	8	15	1 log reduction	in vitro in PBS	82	Read off a graph.
5×10^6 cfu/ml	NaOCl	15 min	10 mg/l free chlorine	10,3	15	”inactivated”	Autoclaved tank water, pH measured after addition of NaOCl.	39	Growth tested on KDM2 agar plate.
5×10^6 cfu/ml	NaOCl	15 min	10 mg/l free	6,3	15	”inactivated”	Distilled water, pH	39	Growth tested on KDM2

			chlorine				measurement after adding NaOCl.		agar plate.
5 x 10^6 cfu/ml	NaOCl	5 min	200 mg/l free chlorine	11,8	15	”inactivated”	Autoclaved tank water, pH measured after addition of NaOCl.	39	Growth tested on KDM2 agar plate.
5 x 10^6 cfu/ml	NaOCl	5 min	200 mg/l free chlorine	12,0	15	”inactivated”	Distilled water, pH measurement after adding NaOCl.	39	Growth tested on KDM2 agar plate.
5 x 10^6 cfu/ml	NaOCl	24 hours	200 mg/l free chlorine	11,8	15	Few survivors.	Autoclaved tank water, pH measured after addition of NaOCl.	39	Growth tested on KDM2 and SKDM agar plates after culture in KDM2 buillon.
5 x 10^6 cfu/ml	NaOCl	15 min	200 mg/l free chlorine	12,0	15	Few survivors.	Distilled water, pH measurement after adding NaOCl.	39	Growth tested on KDM2 and SKDM agar plates after culture in KDM2 buillon.

Conclusion: Under clean condtions in PBS *R. salmoninarum* seem very sensitive to chlorine. In autoclaved tank water 10 mg/l for 15 min or 200 mg/l for 5 min was able to reduce the titer > 3 log.

Streptococcus iniae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	NaOCl	≥ 15 min	3-5 ppm			"powerfull disinfectants"		93	
	Chlorhexidine	≥ 15 min	3-5 ppm			"powerfull disinfectants"		93	

Streptococcus sp.

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	NaOCl	20 min	1600 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other

									papers.
10^7 CFU/ml	NaOCl	60 min	1600 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	20 min	3200 ppm		20	Stable	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	60 min	3200 ppm		20	Stable	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	20 min	200 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	60 min	200 ppm		20	5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200,	58	The actual concentration of disinfectant probably

							400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.		only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Conclusion: According to this paper *Streptococcus* is quite resistant to chlorine!

Vibrio anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^5 cfu/ml	Benzalkonium chlorid	4 min	0,01%		20	> 4 log reduction		85	Concentration of commercial product.
10^5 cfu/ml	Benzalkonium chloride	1 min	0,03%		20	> 4 log reduction		85	Concentration of commercial product.
10^8-10^9 CFU/ml	chloramine-T (SETAX)	1 min	2 g/l		4	≥ 6 log reduction	Sea water added 10% salmon blood.	22	
1,1 x 10^6 CFU/ml	NaOCl	60 min	150 mg/l added			”inactivated”	Test medium: sterilized wastewater from fish slaughterhouse.	48	Tested only at this dose/time combination.
10^5 cfu/ml	NaOCl	2 min	3 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Purelox)
10^5 cfu/ml	NaOCl	1 min	10 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Purelox)
	OCl^-	1 min	0,21 mg/l			>99% reduction		57	Hypoclorite produced by use of batch electrolytic system.
4,5 x 10^6 cfu/ml	OCl^-	1 min	0,07 mg/l			> 4 log reduction	flowrate 3,5 m^3/t, el. 0,5 A	54	Electrolyzed salt water.
5 x 10^4 cfu/ml	OCl^-			8,2		”inactivated”	Sea water, filtrated (10 µm). Flowrate 4 l/min, el. 1,3 A	53	Electrolyzed salt water.

Conclusion: Under loboratory condtions *V. anguillarum* is sensitive to chlorine. In steriliset wastewater from a fish slaugtherhouse 150 mg/l for 60 min was able to reduce the titer to undetectable. No other dose/time combinations were testet.

Vibrio ordalii

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^5 cfu/ml	NaOCl	2 min	3 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Purelox)
10^5 cfu/ml	NaOCl	1 min	10 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Purelox)
10^5 cfu/ml	Benzalkonium chlorid	2 min	0,03%		20	> 4 log reduction		85	Concentration af kommercielt product
10^5 cfu/ml	Benzalkonium chlorid	1 min	0,1%		20	> 4 log reduction		85	Concentration af kommercielt product

Conclusion: Under clean conditions *V. ordalii* is sensitive to chlorine in a low dose.

Vibrio salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^8-10^9 CFU/ml	chloramine-T (SETAX)	1 min	3 g/l		4	6 log reduction	Sea water added 10% salmon blood.	22	

Vibrio sp.

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	NaOCl	20 min	800 ppm		20	5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	NaOCl	60 min	400 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm).	58	The actual concentration of disinfectant probably only half of stated in

							Growht at 20°C and counting already after 24 hours.		article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	20 min	3200 ppm		20	3 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	ClO_2	60 min	3200 ppm		20	3 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	20 min	400 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Quartenary ammonium	60 min	400 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for

									disinfection in this paper is much higher than published in other papers.

Conclusion: According to this paper *Vibrio* is quite resistant to chlorine!

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	chloramine-T	30 min	0,08 - 0,5 % (v/v)		4	> 5 log reduction	Tested at 0,001, 0,01, 0,05, 0,08, 0,1, 0,5, 0,7 and 1%. Hard water, organic loaded.	49	
$10^{6,77}$ cfu/ml	chloramine-T (SETAX)	24 hours	50 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	chloramine-T (SETAX)	24 hours	50 mg/l	8,96	7	1½ log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{6,77}$ cfu/ml	chloramine-T (SETAX)	24 hours	100 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	chloramine-T (SETAX)	24 hours	200 mg/l	8,96	7	3 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{6,77}$ cfu/ml	chloramine-T (SETAX)	24 hours	250 mg/l	7,5		Not detectable (> 6 log reduction)	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	chloramine-T (SETAX)	24 hours	1000 mg/l	8,96	7	4 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
10^{8}-10^{9} CFU/ml	chloramine-T (SETAX)	1 min	2 g/l		4	6 log reduction	Sea water added 10% salmon blood.	22	
10^{3} cells/ml	NaOCl	10 min	0,01 mg/l residual	6,9	20	Stable	Distilled water.	97, 98	Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.
10^{3} cells/ml	NaOCl	30 sec	0,05 mg/l	6,9	20	”inactivated”	Distilled water.	97, 98	Samples tested after ½,

			residual						1, 2, 5, 10, 20 and 30 min.
10^3 cells/ml	NaOCl	10 min	0,05 mg/l residual	6,9	20	Stable	Soft lake water., 30 mg/l $CaCO_3$	97, 98	
10^3 cells/ml	NaOCl	10 min	0,05 mg/l residual	8,2	20	Stable	Hard lake water., 120 mg/l $CaCO_3$	97, 98	
10^3 cells/ml	NaOCl	2 min	0,1 mg/l residual	6,9	20	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$	97, 98	
10^3 cells/ml	NaOCl	2 min	0,1 mg/l residual	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$	97, 98	
$10^{8,28}$ cfu/ml	NaOCl	10 hours	43 mg/l		7	> 3 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
	NaOCl	0,5 hours	48 mg/l			”inactivated”	Fuldskalaforsøg. Kemisk fældet blodvand (Hævning til pH 12, derefter fældning med jernklorid til pH 6,5-7,5)	28	
$10^{6,77}$ cfu/ml	NaOCl	24 hours	50 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{6,77}$ cfu/ml	NaOCl	24 hours	100 mg/l	7,5		Stable	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	NaOCl	30 min	100 mg/l	8,96	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{8,28}$ cfu/ml	NaOCl	10 hours	130 mg/l		7	> 3 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
6 x 10^6 CFU/ml	NaOCl	60 min	200 mg/l added			Vækst		48	Test medium: sterilized wastewater from fish slaughterhouse.
2 x 10^7 CFU/ml	NaOCl	24 hours	200 mg/l added			Vækst		48	Test medium: unsterilized wastewater from fish slaughterhouse, frozen before use.

6×10^6 CFU/ml	NaOCl	60 min	250 mg/l added			”inactivated”		48	Test medium: sterilized wastewater from fish slaughterhouse.
$10^{6,77}$ cfu/ml	NaOCl	2 t	250 mg/l	7,5		> 3 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{6,77}$ cfu/ml	NaOCl	24 hours	250 mg/l	7,5		> 4 log reduction	Full-scale trial (Vikan Akvavet), wastewater from fish slaugtherhouse.	28	Salinity 20 ‰, ”bløggevand” (blood, fish slime and epithelial cells in salt water) diluted with fresh water.
$10^{4,69}$ cfu/ml	NaOCl	2 t	250 mg/l	8,96	7	3 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
2×10^7 CFU/ml	NaOCl	24 hours	250 mg/l added			”inactivated”		48	Test medium: unsterilized wastewater from fish slaughterhouse, frozen before use.
$10^{4,69}$ cfu/ml	NaOCl	24 hours	250 mg/l	8,96	7	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{8,28}$ cfu/ml	NaOCl	10 hours	260 mg/l		7	5 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$6,5 \times 10^7$ CFU/ml	NaOCl	30 min	280 mg/l added			Vækst		48	Testmedium: usteriliseret spildevand fra fiskeslagteri
$6,5 \times 10^7$ CFU/ml	NaOCl	60 min	280 mg/l added			Vækst		48	Testmedium: usteriliseret spildevand fra fiskeslagteri
$6,5 \times 10^7$ CFU/ml	NaOCl	30 min	350 mg/l added			”inactivated”		48	Testmedium: usteriliseret spildevand fra fiskeslagteri
$6,5 \times 10^7$ CFU/ml	NaOCl	60 min	350 mg/l added			”inactivated”		48	Testmedium: usteriliseret spildevand fra fiskeslagteri
$10^{4,69}$ cfu/ml	NaOCl	30 min	350 mg/l	8,96	7	4 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{4,69}$ cfu/ml	NaOCl	2 t	350 mg/l	8,96	7	Not detectable	Full-scale trial (Norskagfisk),	28	Salinity 14-15 ‰

						(≥ 4 log reduction)	blood water from fish slaugtherhouse.		

Conclusion: In full-scale trials under highly contaminated conditions as is found in process wastewater from fish slaugtherhouses 250 mg/l chlorine (administered) as NaOCl for 2 hours were able to decimate *Y. ruckeri* 2 log and after 24 hours to inactivate the bacterium. If the water was pretreated with first pH 12 followed by precipitation with ferrichlorid to pH 6,5 only 50 mg/l chlorine was needed to decimate > 3 log.
When using chloramines-T it was necessary to use a dose of 250-1000 mg/l chlorine for 24 hours to obtain the same degree of inactivation (no pre-treatment of water).

Parasites

Gyrodactylus salaris

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	chlor					følsom		1	From OIE diagnostic manual. No reference stated!

Myxosoma cerebralis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	$Ca(ClO)_2$	14 days	400 ppm		22	Survival	In vitro – spores	42	
	CaOCl	18 hours	1200 ppm			28% of fish infected	Infected mud, chlorine added to water.	44	Control fish 100% infected.
	CaOCl	30 min	10 ppm		12	No spores in fish	Myxosoma cerebralis free in water, fish added after disinfection	44	Control fish 100% infected.
	Cl	14 days	200 ppm		22	Survival	In vitro – spores	42	
	NaOCl	15 min	200 mg/l		15	1 log reduction	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	NaOCl	15 min	500 mg/l		15	5 log reduction	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	NaOCl	15 min	2500 mg/l	8,1	15	100% reduction	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	NaOCl	1 min	131 ppm		room temp.	All dead	in vitro. Triactinomyxon spores	96	

	Quartenary ammonium	14 days	0,1%		22	Survival	In vitro – spores	42	
	Quartenary ammonium	10 min	1000 mg/l		22	1 log reduction	Infectivity of myxospores in tubifex.	37	alkyl dimethyl benzyl ammonium chlorid
	Quartenary ammonium	10 min	1500 mg/l		22	ingen infektion i tubifex	Infectivity of myxospores in tubifex.	37	alkyl dimethyl benzyl ammonium chlorid

Conclusion: Using a concentration of 500-2500 mg/l it will likely be possible to use chlorine for desinfection of *M. cerebralis*.

Trichodina jadranica

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
2,2	chloramine-T	24 hours	50 ppm		25	Survival (0,5)	In vivo, ål	75	Catergorization (category/number of parasites on ell): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000

Conclusion: It seems as if it will be possible to use chlorine for desinfection of Trichodina but the dose has to be bigger than the one used here.

Temperature

Virus

VHSV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^6 $TCID_{50}$/ml	Heating	3 days			20	> 4 log reduction	MEM without serum	51	Read off a graph.
10^6 $TCID_{50}$/ml	Heating	2½ uge			20	app. 4 log reduction	MEM with serum	51	Read off a graph.
10^6 $TCID_{50}$/ml	Heating	3 hours			30	Survival	MEM with serum	51	
10^6 $TCID_{50}$/ml	Heating	24 hours			30	Not detectable	MEM with serum	51	
10^6 $TCID_{50}$/ml	Heating	5 min			50	Survival	MEM with serum	51	
10^6 $TCID_{50}$/ml	Heating	10 min			50	Not detectable	MEM with serum	51	
	Heating	1 hour			60	”inactivated”		20, Dixon (pers. com.)	
10^6 $TCID_{50}$/ml	Heating	1 min			70	Not detectable	MEM with serum	51	

Conclusion: VHSV is heat sensitive and is inactivated at 60°C for 10 min to 1 hour.

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 $TCID_{50}$/ml	Heating	5 hours		7,2	8	Stable	MEM-1 medium	32	
10^7 $TCID_{50}$/ml	Heating	5 hours		7,2	22	Stable	MEM-1 medium	32	
	Heating	8 hours			32	”inactivated”	MEM medium	83	
2,5 x 10^7 $TCID_{50}$/ml	Heating	1 døgn		7,2	32	”inactivated”	MEM-1 medium	32	Karluk Lake isolat
1,5 x 10^7 $TCID_{50}$/ml	Heating	1 døgn		7,2	32	”inactivated”	MEM-1 medium	32	Cedar River isolat
10^7 $TCID_{50}$/ml	Heating	7,3 hours		7,2	32	4 log reduction	MEM-1 medium	32	
	Heating	5 hours		7	35	”inactivated”		100	
2,5 x 10^7 $TCID_{50}$/ml	Heating	140 min		7,2	38	”inactivated”	MEM-1 medium	32	Karluk Lake isolat
1,5 x 10^7 $TCID_{50}$/ml	Heating	140 min		7,2	38	”inactivated”	MEM-1 medium	32	Cedar River isolat
	Heating	20 min		7	40	”inactivated”		100	
	Heating	10 min		7	45	”inactivated”		100	
	Heating	90 sec		7	50	”inactivated”		100	
	Heating	30 sec		7	55	”inactivated”		100	
	Heating	1 hour			60	”inactivated”		20, Dixon (pers. com.)	

Conclusion: IHNV is heat sensitive and is reported inactivated at 55°C for 30 sec and 60°C for 1 hour.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Heating	20 t		7,2	37,5	1 log reduction	MEM-1 medium	32’32	Read off a graph.
	Heating	20 t		7,2	50	2 log reduction	MEM-1 medium	32’32	Read off a graph.
	Heating	< 20 min		7,2	60	95 % reduction		32’32	
$10^{7,2}$ $TCID_{50}$/ml	Heating	30 min		6,8	60	99,9% reduction		74’74	Isolate VR-299
$10^{7,2}$ $TCID_{50}$/ml	Heating	1 hour		3	60	6 log reduction	virus in EMEM with serum	74’74	Isolate VR-299. Result Read off a graph.
$10^{7,2}$ $TCID_{50}$/ml	Heating	4 hours		9	60	6 log reduction	virus in EMEM with serum	74’74	Isolate VR-299. Result Read off a graph.
$10^{7,2}$ $TCID_{50}$/ml	Heating	5 hours		6,8-7	60	6 log reduction	virus in EMEM with and without serum	74’74	Isolate VR-299
	Heating	8 hours		7	60	”inactivated”		100’100	Isolate VR-299
	Heating	16 hours		7,2	60	”inactivated”		32’32	
	Heating	24 hours			60	Survival		20, Dixon (pers. com.)	
	Heating	48 hours			60	”inactivated”		20, Dixon (pers. com.)	
$10^{6,6}$ $TCID_{50}$/ml		1 min			65	Not detectable (> 5 log reduction)	Laboratory trial, 1 part process water + 2 parts	28	

							”bløggevand” from fish slaugtherhouse.		
$10^{5,9}$ $TCID_{50}$/ml	Heating	5 min			65	1½ log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	
	Heating	3,5 hours		7	65	”inactivated”		100˝100	Type VR-299
$10^{5,9}$ $TCID_{50}$/ml	Heating	5 min			70	1,9 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	
	Heating	2 t		7	70	”inactivated”		100˝100	Type VR-299
$10^{5,9}$ $TCID_{50}$/ml	Heating	3 min			75	2,3 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	
	Heating	10 min		7	80	”inactivated”		100˝100	Type VR-299

Conclusion: IPNV is more heat resistant the VHSV and IHNV. The virus is reported to be reduced by 3 log when heated to 60°C for 30 min. Another author reports survival after 24 h at 60°C. In laboratory trials using process water including fish slime, skin scrapings and blood > 5 log reduction were achieved after heating to 65°C for 1 min. In full-scale trials using blood water, 2,3 log reduction were achieved after treatment of IPNV for 3 min.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 x 10^6 $TCID_{50}$/ml	Temperature	14 days			4	Stable	Virus in L15-medium	27	
1 x 10^6 $TCID_{50}$/ml	Temperature	10 days			15	Stable	Virus in L15-medium	27	
1 x 10^6 $TCID_{50}$/ml	Temperature	2 days			37	Survival. 4-5 log reduction	Virus in L15-medium	27	3,2 x 10^1 TCID50/ml på dag 2.
	Heating	2 min			50	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Tested at 45-60°C in 1, 2 and 5 min.
	Heating	1 min			55	”inactivated”	Tissue homogenate of liver, kidney and spleen from moribund ISA-fish, treated and IP-injected in fish.	94	Tested at 45-60°C in 1, 2 and 5 min.
2,5 x 10^6 $TCID_{50}$/ml	Heating	5 min			56	Not detectable	Virus in L15-medium	27	
	Heating	1 hour			60	”inactivated”		20, Dixon (pers. com.)	

Conclusion: ISAV is heat sensitive with reported inactivation times of 56°C for 5 min and 60°C for 1 hour.

KHV

Concentration	Disinfectant	Contact	Concentration	pH	Temp.	Result	Method	Reference	Comments

pathogen		time	disinfectant						
1,6 x 10^4 PFU/ml	Heating	1 min			> 50	Not detectable	Tested at 40, 50, 60 og 70°C i ½, 1, 3 and 5 min.	55	Strain KHV-I

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Temperature	1 year			-20	Stable	Grown virus. Tested after 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	6 months			4	Stable	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	1 year			4	Survival (4-5 log reduction)	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	4 uger			25	Survival (2-3 log reduction)	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	3 months			25	Not detectable	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	1 day			37	Survival (2-3 log reduction)	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
	Temperature	4 days			37	Not detectable	Grown virus. Tested after 1, 4 and 7 days, 4 weeks, 3 and 6 months and 1 year.	30	Isolate: sea bass nodavirus
10 µg purified virus	Heating	30 min		7	50	Not inactivated (0/800 larvae survived, control 230/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
10 µg purified virus	Heating	30 min		7	60	”Effective” (390/800 larvae survived, antigen ELISA negativ - control 230/800)	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV
10^7 $TCID_{50}$/ml	Heating	30 min			60	Not detectable	Hanks balanced salt solution	30	Isolate: sea bass nodavirus
$10^{8\ TCID}{}_{50}$/ml	Heating	30 min			60	6½ log reduction	Hanks balanced salt solution	30	Isolate: sea bass

							med serum		nodavirus
$10^{8\ TCID}{}_{50}$/ml	Heating	1 hour			60	Not detectable	Hanks balanced salt solution med serum	30	Isolate: sea bass nodavirus
	Heating	24 hours			60	”inactivated”		20, Dixon (pers. com.)	

Conclusion: nodavirus is heat sensitive although more resistant than VHSV and IPNV, with reported inactivation times of 1-24 hours at 60°C. Treatment of virus for 30 min at 50°C was not sufficient inhibit disease in an infection trial using day-old striped jack larvae. Increasing the Temperature to 60°C for 30 min was effective in inhibiting disease.

Ranavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Temperature	2 years			-70	Survival	Virus in cell culture medium.	67	EHNV. CPE development slow, 8-10 days
	Temperature	2 years			-70	Survival	Virus in fish tissue.	67	EHNV. CPE development slow, 8-10 days
	Temperature	2 years			-20	Survival	Virus in cell culture medium.	67	EHNV. CPE development slow, 8-10 days
	Temperature	2 years			-20	Survival	Virus in fish tissue.	67	EHNV. CPE development slow, 8-10 days
	Temperature	300 days			4	Survival	Virus in RTG-2 celler	67	EHNV. CPE development slow, 8-10 days
	Heating	24 hours			40	”inactivated”	Virus in cell culture medium.	67	EHNV
	Heating	15 min			60	”inactivated”	Virus in cell culture medium.	67	EHNV
	Heating	24 hours			60	”inactivated”		20, Dixon (pers. com.)	

Conclusion: Ranavirus is reported inactivated by heat treatment for 15 min to 24 hours at 60°C.

Salmonid alphavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Temperature	30 min			4-25	Stable	Heating to 15, 25, 37, 45, 50, 55 and 60°C in 15 min followed by cooling in ice.	79	Salmon pancreas disease virus (SPDV)
	Heating	30 min			37-45	reduced	Heating to 15, 25, 37, 45, 50, 55 and 60°C in 15 min followed by cooling in ice.	79	Salmon pancreas disease virus (SPDV)
	Heating	30 min			50	Not detectable	Heating to 15, 25, 37, 45, 50, 55 and 60°C in 15 min followed by cooling in ice.	79	Salmon pancreas disease virus (SPDV)
	Heating	1 hour			60	”inactivated”		33	SAV1

Conclusion: SAV is reported inactivated at 50°C for 30 min and 60°C for 1 hour.

SVCV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,5}$ $TCID_{50}$/ml	Temperature	180 days			-74	2 log reduction	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{7,8}$ $TCID_{50}$/ml	Temperature	180 days			-74	Stable	Cellekulturmedium med 5% serum. Titreret day 3, 7, 14, 21, 28, 75, 110 og 180 days	3	
$10^{6,5}$ $TCID_{50}$/ml	Temperature	180 days			-20	> 3½ log reduction	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{7,8}$ $TCID_{50}$/ml	Temperature	180 days			-20	2 log reduction	Cellekulturmedium med 5% serum. Titreret day 3, 7, 14, 21, 28, 75, 110 og 180 days	3	
$10^{6,5}$ $TCID_{50}$/ml	Temperature	110 days			4	> 4 log reduction	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{6,5}$ $TCID_{50}$/ml	Temperature	180 days			4	”inactivated”	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{7,8}$ $TCID_{50}$/ml	Temperature	180 days			4	> 3 log reduction	Cellekulturmedium med 5% serum. Titreret day 3, 7, 14, 21, 28, 75, 110 og 180 days	3	
$10^{6,5}$ $TCID_{50}$/ml	Temperature	7 days			22-24	2 log reduction	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{6,5}$ $TCID_{50}$/ml	Temperature	14 days			22-24	”inactivated”	Cell culture medium without serum. Titrated day 3, 7, 14, 21, 28, 75, 110 and 180.	3	
$10^{7,8}$ $TCID_{50}$/ml	Temperature	21 days			22-24	> 3 log reduction	Cellekulturmedium med 5% serum. Titreret day 3, 7, 14, 21, 28, 75, 110 og 180 days	3	
$10^{7,8}$ $TCID_{50}$/ml	Temperature	75 days			22-24	”inactivated”	Cellekulturmedium med 5% serum. Titreret day 3, 7, 14, 21, 28, 75, 110 og 180 days	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	480 min			30	½ log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	

$10^{7,5}$ $TCID_{50}$/ml	Heating	480 min			35	1 log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	240 min			40	3 log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	480 min			40	> 4 log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	60 min			45	3 log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	180 min			45	> 5 log reduction	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
$10^{7,5}$ $TCID_{50}$/ml	Heating	60 min			50	”inactivated”	Cell cultur medium with 5% serum. Titrated after 5, 10, 20, 30, 60, 120, 240 and 480 min.	3	
	Heating	1 hour			60	”inactivated”		20, Dixon (pers. com.)	

Conclusion: SVCV is inactivated at ≤ 60°C after 1 h.

Channel catfish virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Heating	24 hours			60	”inactivated”		20, Dixon (pers. com.)	

Bacteria

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,4 x 10^6 cfu/ml	Heating	48 hours		7	35	”inactivated”		100	

1,4 x 10^6 cfu/ml	Heating	3 hours		7	40	”inactivated”		100	
1,4 x 10^6 cfu/ml	Heating	10 min		7	45	”inactivated”		100	
1,4 x 10^6 cfu/ml	Heating	2 min		7	50	”inactivated”		100	
	Heating	1 hour			60	Survival		20, Dixon (pers. com.)	

Conclusion: *A. salmonicida* is reported inactivated after heat treatment for 2 min at 50°C. This is disputed by another report stating survival after 1 hour at 60°C.

Lactococcus garviae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Heating	24 hours			60	Survival		20, Dixon (pers. com.)	
	Heating	48 hours			60	”inactivated”		20, Dixon (pers. com.)	

Vibrio anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^6 CFU/ml	Heating	1 min			60	”inactivated”		48	Test medium: sterilized wastewater from fish slaughterhouse.
10^7 CFU/ml	Heating	2 min			60	Growth		48	Test medium: unsterilized wastewater from fish slaughterhouse.
	Heating	1 hour			60	Survival		20, Dixon (pers. com.)	
10^6 CFU/ml	Heating	15 sec			72	”inactivated”		48	Test medium: sterilized wastewater from fish slaughterhouse.
10^7 CFU/ml	Heating	15 sec			72	”inactivated”		48	Test medium: unsterilized wastewater from fish slaughterhouse.

Mycobacterium chelonei

Concentration	Disinfectant	Contact	Concentration	pH	Temp.	Result	Method	Reference	Comments

pathogen		time	disinfectant						
7,5 x 10^6 cfu/ml	Heating	24 hours		7	40	”inactivated”		100	
7,5 x 10^6 cfu/ml	Heating	4 hours		7	45	”inactivated”		100	
7,5 x 10^6 cfu/ml	Heating	60 min		7	50	”inactivated”		100	
7,5 x 10^6 cfu/ml	Heating	15 min		7	55	”inactivated”		100	
7,5 x 10^6 cfu/ml	Heating	2,5 min		7	60	”inactivated”		100	
	Heating	6 hours			60	”inactivated”		20, Dixon (pers. com.)	
7,5 x 10^6 cfu/ml	Heating	< 30 sec		7	65	”inactivated”		100	

Photobacterium damselae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Heating	1 hour			60	Survival		20, Dixon (pers. com.)	

Renibacterium salmoninarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
≥ 10^5 cfu/ml	Heating	> 2 t		7	40	”inactivated”		100	
≥ 10^5 cfu/ml	Heating	> 6 t		7	45	”inactivated”		100	
≥ 10^5 cfu/ml	Heating	> 4 hours		7	50	”inactivated”		100	
≥ 10^5 cfu/ml	Heating	> 3 hours		7	55	”inactivated”		100	
	Heating	1 hour			60	”inactivated”		20, Dixon (pers. com.)	
≥ 10^5 cfu/ml	Heating	> 15 min		7	65	”inactivated”		100	

Streptococcus iniae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Heating	5 min			60	”inactivated”		20, Dixon (pers. com.)	

Yersinia ruckeri

Concentration	Disinfectant	Contact	Concentration	pH	Temp.	Result	Method	Reference	Comments

pathogen		time	disinfectant						
6,6 x 10^7 CFU/ml	Heating	1 min			60	”inactivated”		48	Test medium: sterilized wastewater from fish slaughterhouse.
6,5 x 10^7 CFU/ml	Heating	2 min			60	”inactivated”		48	Test medium: unsterilized wastewater from fish slaughterhouse.
	Heating	1 hour			60	Survival		20, Dixon (pers. com.)	
$10^{8,28}$ cfu/ml	Heating	1 min			65	5 log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{4,69}$ cfu/ml	Heating	3 min		8,96	65	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{8,28}$ cfu/ml	Heating	5 min			65	7½ log reduction	Laboratory trial, 1 part process water + 2 parts ”bløggevand” from fish slaugtherhouse.	28	
$10^{5,04}$ cfu/ml	Heating	24 hours			65	Stable	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	
$10^{4,69}$ cfu/ml	Heating	1 min		8,96	70	4 log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{5,04}$ cfu/ml	Heating	24 hours			70	Stable	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	
6,6 x 10^7 CFU/ml	Heating	15 seconds			72	”inactivated”		48	Test medium: sterilized wastewater from fish slaughterhouse.
6,5 x 10^7 CFU/ml	Heating	15 sec			72	”inactivated”		48	Test medium: unsterilized wastewater from fish slaughterhouse.
$10^{4,69}$ cfu/ml	Heating	1 min		8,96	75	3½ log reduction	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{4,69}$ cfu/ml	Heating	2 min		8,96	75	Not detectable (≥ 4 log reduction)	Full-scale trial (Norskagfisk), blood water from fish slaugtherhouse.	28	Salinity 14-15 ‰
$10^{5,04}$ cfu/ml	Heating	24 hours			75	Not detectable (> 6 log reduction)	Full-scale trial (Norskagfisk), blood water from fish	28	

							slaugtherhouse.		

Parasites

Gyrodactylus salaris

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
75	Temperature	60 hours			3	All dead	Free, without host.	80	
422	Temperature	365 hours			3	All dead	On dead host.	80	
88	Temperature	45 hours			12	All dead	Free, without host.	80	
315	Temperature	142 hours			12	All dead	On dead host.	80	
65	Temperature	27 hours			18	All dead	Free, without host.	80	
204	Temperature	72 t			18	All dead	On dead host.	80	

Myxosoma cerebralis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Temperature	7 days			-80	No infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	Temperature	2 months			-80	No infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore in head tissue from fish.
	Temperature	7 days			-20	No infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore suspension
	Temperature	2 months			-20	No infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore in head tissue from fish.
96-192	Temperature	105 min			-20	Survival?	in vitro. Triactinomyxon spores	96	1,0 ± 0.8% probably alive, remaining dead.
	Temperature	2 months			4	Active infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore in head tissue from fish.
	Temperature	7 days			5	Active infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore suspension
45-131	Temperature	105 min			7	Alive 31%, dead 46%	in vitro. Triactinomyxon spores	96	
51-124	Temperature	60 min			19-21	Alive 72%, dead 5%	in vitro. Triactinomyxon spores	96	
	Temperature	2 months			20	No infection in tubifex.	Infectivity of myxospores in tubifex.	37	Myxospore in head tissue from fish.
	Temperature	7 days			22	Active infection in	Infectivity of myxospores in	37	Myxospore suspension

						tubifex.	tubifex.		
	Heating	5 min			58	Survival	in vitro. Triactinomyxon spores	96	
100 spores talt	Heating	10 min			70	60% dead	In vitro - spores farvet med methylenblå som tegn på død (skal eftervises)	43	
	Heating	5 min			75	All dead	in vitro. Triactinomyxon spores	96	
100 spores talt	Heating	10 min			80	98% dead	In vitro - spores farvet med methylenblå som tegn på død (skal eftervises)	43	4 trials where all dead, 1 trial where 88% dead.
100 spores talt	Heating	10 min			90	All dead	In vitro - spores farvet med methylenblå som tegn på død (skal eftervises)	43	

Percolation

It has not been possible to find any references describing the decimating effect of percolating of fish pathogenic viruses. Furthermore it has not been possible to find publications describing the effect of percolating other birnaviruses. It is therefore not possible to validate if this procedure is safe to use.

Other procedures:

Iodine based disinfectants

Virus

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{4,53}$ - $10^{5,18}$ pfu/ml	Povidon iodine	7,5 seconds	0,10 mg/l free iodide			99,49 → 99,99% reduction	Distilled water.	11	6 isolates representing 5 types.
$10^{4,62}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide	6		> 99,99% reduction		11	Testing of effect of pH.
$10^{4,66}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide	7		> 99,99% reduction		11	Testing of effect of pH.
$10^{4,74}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual	8		99,89% reduction		11	Testing of effect of pH.

			free iodide						
$10^{4,71}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide	9		90,78% reduction		11	Testing of effect of pH.
	Povidon iodine	5 min	0,4 mg/l residual free iodide			> 4 log reduction for alle saliniteter	Natural seawater containg 0, 4, 7½, 15½ and 32 ‰ salte.	11	Effect of BSA (dirty conditions).
$10^{5,04}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide			> 4 log reduction for alle saliniteter	Dirty conditions: iodide + calf serum(0,002%) mixed before virus added	11	Effect of BSA (dirty conditions).
$10^{5,04}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide			Stable	Dirty conditions: iodide + calf serum(0,016%) mixed before virus added	11	Effect of BSA (dirty conditions).
$10^{4,81}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide			> 4 log reduction for alle saliniteter	Dirty conditions: virus + calf serum (0,002%) mixed before iodide added	11	Effect of BSA (dirty conditions).
$10^{4,81}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide			99,96% reduction	Dirty conditions: virus + calf serum (0,016%) mixed before iodide added	11	Effect of BSA (dirty conditions).
$10^{4,81}$ pfu/ml	Povidon iodine	5 min	0,4 mg/l residual free iodide			66,67% reduction	Dirty conditions: virus + calf serum (0,064%) mixed before iodide added	11	Effect of BSA (dirty conditions).
$10^{4,41}$ - $10^{4,91}$ pfu/ml	Povidon iodine	7,5 sec	0,4 mg/l residual free iodide			Not detectable til Stable	Natural water from 8 different sources (fresh + salt)	11	
$10^{4,41}$ - $10^{4,91}$ pfu/ml	Povidon iodine	7,5 sec	0,8 mg/l residual free iodide			≥ 4 log reduction	Natural water from 8 different sources (fresh + salt)	11	
	Iodophor	5 min	8 ppm	6,0		”inactivated”		8	1 ppm = 1 mg/l
	Iodophor	30 sec	12 ppm	7,0		”inactivated”		8	
	Iodophor	15 sec	25 ppm	7,0		”inactivated”		8	
	Iodophor	5 min	32 ppm	8,6		”inactivated”		8	
10^{6} PFU/ml	Iodophor	10 min	100 mg/l			> 3 log reduction	Green eggs and eyed eggs treated in 10 or 60 min	31	

Conclusion: Under laboratory conditions IHNV is sensitive to disinfection with iodine. The higher the pH and the more organic waste the more iodine is needen to disinfect the same amount of virus. Recommended dose: 100 ppm, 10 min contact time.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{7,41}$ $TCID_{50}$/ml	Iodophor, acid	30 min	0,033% (v/v)		4	> 4 log reduction	Tested at 0,0055, 0,011, 0,022,0,033, 0,044 and 0,055%. Hard water, Dirty	49	

							conditions.		
$10^{3,9}$ $TCID_{50}$/ml	Iodophor	5 min	4 mg/l residual		21	”inactivated”	Testet using ½, 1,2, 4, 8, 16, 32 and 64 mg/l residual. Distilled water.	23	IPNV: Serotype Buhl. Iodophor: Betadine
$10^{3,8\ TCID}_{50}$/ml	Iodophor	15 seconds	12 mg/l residual		21	”inactivated”	Tested at time 0, 15, 30, 60, 120 s. Distilled water.	23	IPNV: Serotype Buhl. Iodophor: Betadine
$10^{3,9}$ $TCID_{50}$/ml	Iodophor	5 min	16 mg/l residual	6-8,6	21	”inactivated”	Testet using ½, 1,2, 4, 8, 16, 32 and 64 mg/l residual. Distilled water.	23	IPNV: Serotype Buhl. Iodophor: Betadine
$10^{5,5\ TCID}_{50}$/ml	Iodophor	5 min	30 ppm iodine		room temp.	”inactivated”	PBS	17	Wescodyne
	Iodophor	5 min	32 ppm	6,9		”inactivated”		8	
$10^{6,6}$ $TCID_{50}$/ml	Iodophor	5 min	35 ppm iodine		room temp.	”inactivated”	PBS	17	Wescodyne
$10^{6,6}$ $TCID_{50}$/ml	Iodophor	3 min	45 ppm iodine		room temp.	Survival	PBS	17	Wescodyne
	Actomar	5 min	0,01%			> 4 log reduction		5	
$10^{5,2}$ $TCID_{50}$/ml	Actomar	20 min	50 ppm			”inactivated”	Without serum	6	Active iodide 50, 100, 150 and 200 ppm testet. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{5,2}$ $TCID_{50}$/ml	Actomar	2 min →	50 ppm			3 log reduction	With 5 % serum	6	Active iodide 50, 100, 150 and 200 ppm testet. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{5,8\ TCID}_{50}$/ml	Actomar	6 min	150 ppm			3 log reduction	With 5 % serum	37	Active iodide 50, 100, 150 and 200 ppm testet. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{5,8\ TCID}_{50}$/ml	Actomar	20 min	150 ppm			”inactivated”	With 5 % serum	6	Active iodide 50, 100, 150 and 200 ppm testet. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
	iodophor	5 min	80-100 ppm		5	> 99,9%	Iodophor added virus	24	FAM (acid iodophor)
	iodophor	5 min	80-100 ppm		5	> 99,9%	Iodophor added virus	24	Buffodine (neutral iodophor)
	iodophor	5 min	80-100 ppm		5	90%	Eggs before hardening, surface infected with virus	24	Buffodine (neutral iodophor)

Conclusion: Under laboratory conditions IPNV is sensitive for iodine. When conditions are dirty more iodine is needed. Recommended dose for 3 log reduction: 150 ppm, 10 min contact time.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
5,1 log10 ffu/ml	Iodophor	5 min	50 ppm		4	> 4,8 log reduction	Hard water, 342 ppm total hardness.. Testet with 50, 100 and 200 ppm.	89	Buffodine
5,1 log10 ffu/ml	Iodine	5 min	100 ppm		4	> 4,8 log reduction	Hard water, 342 ppm total hardness, with and without addition of serum.	89	Tegodyne
5,5 log10 ffu/ml	Iodine	5 min	100 ppm		4	> 5,2 log reduction	Hard water, 342 ppm total hardness, with and without addition of serum. Testet with 100, 200 and 400 ppm.	89	FAM 30

Conclusion: Under laboratory conditions ISAV is sensitive for iodine. Recommended dose: 100 ppm, 5 min contact time.

VHSV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Iodophor	5 min	8 ppm	6,9		”inactivated”		8	
10^7 $TCID_{50}$/ml	Iodophor	1 min	50 ppm		15	Not detectable	Diluted in PBS. 1% fetal calf serum.	65	Isolat JF001Ehi1. Dilution scale 1:50. Isodine.
10^7 $TCID_{50}$/ml	Iodophor	1 min	50 ppm		15	Not detectable	Fortyndet i kunstig havvand. 1% føtalt calf serum.	65	Isolat JF001Ehi1. Dilution scale 1:50. Isodine.
$10^{5,8\ TCID}_{50}$/ml	Actomar	8 min →	50 ppm			3 log reduction	With 5 % serum	6	Active iodide, 50 and 100 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{5,5\ TCID}_{50}$/ml	Actomar	4 min	50 ppm			”inactivated”	Without serum	6	Active iodide, 50 and 100 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
	Actomar	5 min	100 ppm			”inactivated”		5	Author claim 100% reduction.
$10^{6,5\ TCID}_{50}$/ml	Actomar	2 min	100 ppm			”inactivated”	With 5 % serum	6	Active iodide, 50 and 100 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
10^8 pfu/ml	Iodophor	10 min	100 ppm			Not detectable	fiskeæg added virus	95	genotype IVb
	iodophor	5 min	80-100 ppm		5	> 99,9%	Iodophor added virus	24	Buffodine (neutral

									iodophor)
	iodophor	5 min	80-100 ppm		5	> 99,99%	Nystrøgne, Not hærdede æg overflade inficeret med virus	24	Buffodine (neutral iodophor)
	iodophor	5 min	80-100 ppm		5	> 99,9%	Iodophor added virus	24	FAM (acid iodophor)

Conclusion: Iodophores can inactivate VHSV on fish eggs using a dose of 100 ppm and a contact time of 10 min. The results indicate that iodine will be used under dirty conditions minimizing the disinfecting effect. Recommended dose: 100 ppm, 10 min.

SVCV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Actomar	5 min	100 ppm			99 % reduction		5	
$10^{7,2}$ $TCID_{50}$/ml	Actomar	10 min	100 ppm			"inactivated"	Without serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{7,5\ TCID}_{50}$/ml	Actomar	2 min og fremefter	100 ppm			2 log reduction	With 5 % serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{7,8\ TCID}_{50}$/ml	Actomar	4 min	150 ppm			3 log reduction	With 5 % serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{7,5\ TCID}_{50}$/ml	Actomar	10 min	200 ppm			"inactivated"	With 5 % serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.

Conclusion: The results indicates that a dose of 100 ppm wil be used under dirty conditions rendering an acceptable disinfection impossible. Recommended dose: 200 ppm, 10 min.

PFRV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Actomar	5 min	100 ppm			99,99 % reduction		5	
$10^{7,5\ TCID}_{50}$/ml	Actomar	4 min	100 ppm			"inactivated"	Without serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{7,5\ TCID}_{50}$/ml	Actomar	4 min og fremefter	100 ppm			6 log reduction	With 5 % serum	6	Active iodide 100, 150 and 200 ppm tested.

									Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.
$10^{7,2}$ $TCID_{50}$/ml	Actomar	10 min	150 ppm			"inactivated"	With 5 % serum	6	Active iodide 100, 150 and 200 ppm tested. Time 2, 4, 5, 6, 8, 10, 20 and 30 min tested.

Conclusion: The results indicate that a dose of 100 ppm will be used under dirty conditions, but the obtained reduction was still satisfactory.

KHV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 - 1,5 x 10^4 PFU/ml	Iodophor	30 seconds	130 mg/l		0	Not detectable	Virus and disinfectant mixed 1:1, tested after 30 sec and 20 min. Diluted 1:10 using L15 medium and 200 µl inoculated .	55	Strain KHV-I. The method cannot detect a 3 log reduction.
1 - 1,5 x 10^4 PFU/ml	Iodophor	30 seconds	200 mg/l		25	Not detectable	testet ved 30 sec og 20 min.	55	Strain KHV-I. The method cannot detect a 3 log reduction.

Conclusion: Although the method used is not capable of detecting a 3 log reduction the results indicate the KHV is sensitive to disinfection using iodophores.

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Jod	15 min	40 mg/l			Not inactivated (all larvae dead after 15 days)	Washing of eggs from noda infected ovaries in seawater, followed after hatching.	9	Isolate: SJNNV
10 µg purified virus	Jod	10 min	50 mg/ml		20	"Effective"	Diluted in 1 ml PBS. Used for infection trial in day old striped jack larvae. Concentration testet: 2,5 - 100 mg/ml.	9	Isolate: SJNNV
$10^{6,125}$ $TCID_{50}$/ml	iodophor, buffered	5 min	25 ppm I_2		15	Not detectable	Distilled water. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,125}$ $TCID_{50}$/ml	iodophor, buffered	30 min	100 ppm I_2		15	4½ log reduction	HBSS + calf serum. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus

Conclusion: Under laboratory conditions nodavirus is sensitive to disinfection with iodine products but it seems that nodavirus is a bit more resistant than VHSV and IHNV. Recommended concentration: 100 ppm, 30 min.

Oncorhynchus masou virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Iodophor	30 sec	40 ppm		0	”inactivated”		35	
	Iodophor	30 sec	40 ppm		25	”inactivated”		35	

Bacteria

Aeromonas liquefaciens

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 x 10^7/ml	Iodophor	30 sec	25 ppm	7	10-13	> 5 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1 x 10^7/ml	Iodophor	120 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1 x 10^6/ml	Iodophor	300 sec	25 ppm	8	10-13	> 5 log reduction	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1 x 10^7/ml	Povidon iodine	15 sec	25 ppm	7	10-13	≥ 6 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
1 x 10^7/ml	Povidon iodine	120 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
1 x 10^7/ml	Povidon iodine	300 sec	25 ppm	8	10-13	> 5 log reduction	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine

Conclusion: Under clean conditions 25 ppm for a few minutes will provide an aceptable reduction.

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	I_2		1,0 mg/l added	7,2	7	99,9% reduction	PBS	71	Reduction rate: 0,21/sec
10^7 CFU/ml	I_2	36 sec	1 mg/l added	7,2	7	99,9 % reduction	PBS	71	
10^7 CFU/ml	I_2		1,3 mg/l added	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,14/sec
10^7 CFU/ml	I_2	1 min	1,3 mg/l added	7,8	7	3½ log reduction	Wastewater from aquaculture	71	Read off a graph.

							(15,7 ‰ salinity).		
10^7 CFU/ml	I_2		2,6 mg/l added	7,8	7	99,9% reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,26/sec
10^7 CFU/ml	I_2	40 sec	2,6 mg/l added	7,8	7	4½ log reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Read off a graph.
	Iodophor, acid	30 min	0,28 % (v/v)		4	> 5 log reduction	Tested at 0,16, 0,2, 0,28, 0,4, 0,8, 1, 1,6, 2 and 3,2%. Hard water, high organic load.	49	
2,6 x 10^7/ml	Iodophor	15 sec	25 ppm	7	10-13	6 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
4 x 10^7/ml	Iodophor	60 sec	25 ppm	8	10-13	Not detectable (> 7 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
2,6 x 10^7/ml	Iodophor	120 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
10^5 cfu/ml	Povidon iodine	3 min	10 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Isodine)
10^8 cfu/ml	Povidon iodine	5 min	5 ppm		20	Stable	Test of effect of bacteria titer.	85	Concentration of commercial product. (Isodine)
10^6 cfu/ml	Povidon iodine	5 min	5 ppm		20	> 3 log reduction	Test of effect of bacteria titer.	85	Concentration of commercial product. (Isodine)
10^4 cfu/ml	Povidon iodine	1 min	5 ppm		20	> 3 log reduction	Test of effect of bacteria titer.	85	Concentration of commercial product. (Isodine)
$10^{5,5}$ cfu/ml	Povidon iodine	4 min	5 ppm		20	Stable	10 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	Povidon iodine	4 min	5 ppm		20	4½ log reduction	0 ppm calf serum added.	85	
$10^{5,5}$ cfu/ml	Povidon iodine	3 min	5 ppm		5	3½ log reduction		85	Test of effect of Temperature.
$10^{5,5}$ cfu/ml	Povidon iodine	3 min	5 ppm		25	4½ log reduction		85	Test of effect of Temperature.
$10^{5,5}$ cfu/ml	Povidon iodine	5 min	5 ppm		15	Stable	Artificial sea water.	85	
$10^{5,5}$ cfu/ml	Povidon iodine	1-3 min	5 ppm		15	3½ - 4 log reduction	Hard water (300 ppm $CaCO_3$)	85	
$10^{5,5}$ cfu/ml	Povidon iodine	1-3 min	5 ppm		15	3½ - 4 log reduction	Distilled water.	85	
1 x 10^7/ml	Povidon iodine	15 sec	25 ppm	7	10-13	> 5 log reduction for 3 of 4 strains	Distilled water. 4 strains tested. Time: 15, 30, 60, 120	84	Betadine

							and 300 seconds.		
1 x 10^7/ml	Povidon iodine	300 sec	25 ppm	7	10-13	Not detectable	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
4 x 10^7/ml	Povidon iodine	300 sec	25 ppm	7	10-13	Not detectable	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
10^5 cfu/ml	Povidon iodine	1 min	30 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Isodine)

Conclusion: The lower the Temperature, the highter the titer of the pathogen, the more organic dirt, the worse the obtaine disinfection. The results indicate that a dose of 25 ppm for 5 min will provide ≥ 3 log reduction.

Carnobacterium piscicola

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Iodophor, acid	30 min	0,4 - 1% (v/v)		4	> 5 log reduction	Tested at 0,16, 0,2, 0,28, 0,4, 0,8, 1, 1,6, 2 and 3,2%. Hard water, high organic load.	49	

Flexibacter columnaris

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,7 x 10^6/ml	Iodophor	15 sec	25 ppm	7	10-13	> 5 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1,7 x 10^6/ml	Iodophor	300 sec	25 ppm	7	10-13	Not detectable (> 6 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
4 x 10^4/ml	Iodophor	30 sec	25 ppm	8	10-13	Not detectable (> 3 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
2 x 10^6/ml	Povidon iodine	15 sec	25 ppm	7	10-13	Not detectable (> 6 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
2 x 10^6/ml	Povidon iodine	120 sec	25 ppm	7	10-13	Not detectable (≥ 6 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
4 x 10^4/ml	Povidon iodine	120 sec	25 ppm	8	10-13	Not detectable (> 3	Distilled water. 1 strain	84	Betadine

						log reduction)	tested. Time: 15, 30, 60, 120 and 300 seconds.		

Conclusion: Under clean conditions 25 ppm for 5 min will inactivate the bacteria.

Cytophaga psychrophila (Flavabacterium psychrophilum)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1,4 x 10^6/ml	Iodophor	15 sec	25 ppm	7	10-13	> 5 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1,4 x 10^6/ml	Iodophor	60 sec	25 ppm	7	10-13	Not detectable (> 6 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
5 x 10^3/ml	Iodophor	15 sec	25 ppm	8	10-13	Not detectable (> 3 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1,4 x 10^6/ml	Povidon iodine	15 sec	25 ppm	7	10-13	5 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
1,4 x 10^6/ml	Povidon iodine	30 sec	25 ppm	7	10-13	≥ 6 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
5 x 10^3/ml	Povidon iodine	30 sec	25 ppm	8	10-13	Not detectable (> 3 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine

Conclusion: Under clean conditions 25 ppm for 5 min will inactivate the bacteria.

Edwardsiella tarda

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	Povidon iodine	20 min	800 ppm		20	5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Povidon iodine	60 min	800 ppm		20	5 log reduction	Dilution 1:1 of bacteria and	58	The actual concentration

							disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.		of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Conclusion: this paper states that *E. tarda* is much more resistant to iodine that other bacteria!

Kidney disease (Corynebacterium sp. - Renibacterium salmoninarum?)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
Growth	Iodophor	15 sec	25 ppm	7	10-13	Not detectable	Distilled water. 2 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
Growth	Iodophor	300 sec	25 ppm	8	10-13	Not detectable	Distilled water. 1 strain tested. Time: 300 seconds.	84	Wescodyne
Growth	Povidon iodine	15 sec	25 ppm	7	10-13	Not detectable	Distilled water. 2 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
Growth	Povidon iodine	300 sec	25 ppm	8	10-13	Not detectable	Distilled water. 1 strain tested. Time: 300 seconds.	84	Betadine

Renibacterium salmoninarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
> 1667 Bacteria/æg	Povidon iodine	15 min	500 mg/l		15	166/170 (97,6%) of the eggs sterile on surface	Eggs from infected coho salmon.	25	

Conclusion: Disinfection of salmon egg seems to require a higher amount of iodine to be disinfected that do the bacteria under clean conditions (destilled water). Under clean conditions *R. salmoninarum* is comparable to other fish pathogenic bacteria requiring 25 ppm for 5 min. A dose of 500 mg/l did not completely inactivate *R. salmoniarum* on the surface of the eggs though most of the eggs were rendered sterile.

Lactococcus garviae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Iodophor, acid	30 min	0,4 % (v/v)		4	> 5 log reduction	Tested at 0,16, 0,2, 0,28, 0,4,	49	

							0,8, 1, 1,6, 2 and 3,2%. Hard water, high organic load.		

Streptococcus sp

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	Povidon iodine	20 min	3200 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Povidon iodine	60 min	1600 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Conclusion: this paper states that *Streprococcus* is much more resistant to iodine that other bacteria!

Vibrio anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^5 cfu/ml	Povidon iodine	1 min	10 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Isodine)
3 x 10^7/ml	Iodophor	15 sec	25 ppm	7	10-13	> 6 log reduction	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
3 x 10^7/ml	Iodophor	120 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
5 x 10^6/ml	Iodophor	60 sec	25 ppm	8	10-13	Not detectable (> 6 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
1 x 10^5/ml	Povidon iodine	15 sec	25 ppm	7	10-13	Not detectable (> 5 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine

5 x 10^6/ml	Povidon iodine	300 sec	25 ppm	8	10-13	Not detectable (> 6 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine

Conclusion: Under clean conditions 25 ppm for 5 min will inactivate the bacteria.

Vibrio ordalii

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^5 cfu/ml	Povidon iodine	1 min	30 ppm		20	> 4 log reduction		85	Concentration of commercial product. (Isodine)

Conclusion: Under clean conditions 25 ppm for 5 min will inactivate the bacteria.

Vibrio sp.

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	Povidon iodine	20 min	1600 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	Povidon iodine	60 min	800 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Conclusion: this paper states that *Vibrio* is much more resistant to iodine than stated in other papers!

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
3 x 10^7/ml	Iodophor	15 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
5 x 10^6/ml	Iodophor	15 sec	25 ppm	8	10-13	Not detectable (> 6 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne
	Iodophor, acid	30 min	0,28 % (v/v)		4	> 5 log reduction	Tested at 0,16, 0,2, 0,28, 0,4, 0,8, 1, 1,6, 2 and 3,2%. Hard water, high organic load.	126	
2 x 10^7/ml	Povidon iodine	15 sec	25 ppm	7	10-13	Not detectable (> 7 log reduction)	Distilled water. 4 strains tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
5 x 10^6/ml	Povidon iodine	15 sec	25 ppm	8	10-13	Not detectable (> 6 log reduction)	Distilled water. 1 strain tested. Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine

Conclusion: Under clean conditions 25 ppm for 5 min will inactivate the bacteria.

Parasites

Gyrodactylus salaris

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Iodine					Sensitive		1	From OIE diagnostic manual. No reference stated!

Myxosoma cerebralis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Povidon iodine	10 min	500 ppm		room temp.	Survival	in vitro. Triactinomyxon spores	96	5% of commercial product.

	Povidon iodine	10 min	5000 ppm		room temp.	Survival	in vitro. Triactinomyxon spores	96	50% of commercial product.

Fungae

Phoma herbarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
Growth	Povidon iodine	120 sec	25 ppm	7	10-13	No growth	Distilled water. 1 strain tested (spores). Time: 15, 30, 60, 120 and 300 seconds.	84	Betadine
Growth	Iodophor	120 sec	25 ppm	7	10-13	No growth	Distilled water. 1 strain tested (spores). Time: 15, 30, 60, 120 and 300 seconds.	84	Wescodyne

Saprolegnia parasitica

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
Growth	Povidon iodine	300 sec	25 ppm	7	10-13	Growth	Distilled water.. 1 stamme testet (mycelium). Tid: 15, 30, 60, 120 og 300 seconds.	84	Betadine
Growth	Iodophor	300 sec	25 ppm	7	10-13	Growth	Distilled water.. 1 stamme testet (mycelium). Tid: 15, 30, 60, 120 og 300 seconds.	84	Wescodyne

Ozone

Virus

VHSV

It has not been possible to find any references.

IHNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^4 - $10^5$$TCID_{50}$/ml	ozone	30 sec	total residual	6,9	10	”inactivated”	Ozone tilført: 70 mg/h/L,	99, 98	C T value: 0,3 mg*s/l

			oxidants 0,01 mg/l				Distilled water.		
	ozone	15 sec	total residual oxidants 0,5 mg/l			99% infectivity reduction		102	C T value: 7,5 mg*s/l
10^4 - 10^5TCID$_{50}$/ml	ozone	10 min	70 mg/h/L	6,9	10	”inactivated”	Not possible to obtain a stable ozone residual. Soft lake water, 30 mg/l $CaCO_3$.	99, 98	
10^4 - 10^5 TCID$_{50}$/ml	ozone	10 min	70 mg/h/L	8,2	10	”inactivated”	Not possible to obtain a stable ozone residual. Hard lake water, 120 mg/l $CaCO_3$.	99, 98	

Conclusion: IHNV is sensitive to treatment with ozone. Based on these figures the dose needed for a 3 log reduction is unknown.

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^5 TCID$_{50}$/ml	ozone	60 sec	total residual oxidants 0,01 mg/l	6,9	10	”inactivated”	Ozone added: 70 mg/h/L, Distilled water.	99, 98	C T value: 0,6 mg*s/l
$10^{5,5}$ TCID$_{50}$/ml	ozone	60 sec	0,20 mg/l		9-12	> 4 log reduction	Lake water, autoclaved.	70	C T value: 12 mg*s/l
$10^{5,5}$ TCID$_{50}$/ml	ozone	120 sec	0,20 mg/l		9-12	> 5 log reduction	Lake water, autoclaved.	70	C T value: 24 mg*s/l
$10^{5,5}$ TCID$_{50}$/ml	ozone	60 sec	0,20 mg/l		9-12	> 5 log reduction	Brackish water, salinity 15 ‰, autoclaved.	70	C T value: 12 mg*s/l
$10^{5,5}$ TCID$_{50}$/ml	ozone	60 sec	0,20 mg/l		9-12	> 5 log reduction	Sea water, salinity 32 ‰, autoclaved.	70	C T value: 12 mg*s/l
	ozone	1 min	total residual oxidants 0,5 mg/l			99% infectivity reduction		102	C T value: 30 mg*s/l
10^5 TCID$_{50}$/ml	ozone	30 sec	90 mg/h/L	6,9	10	”inactivated”	Not possible to obtain a stable ozone residual. Soft lake water, 30 mg/l $CaCO_3$.	99, 98	
10^5 TCID$_{50}$/ml	ozone	10 min	90 mg/h/L	8,2	10	”inactivated”	Not possible to obtain a stable ozone residual. Hard lake water, 120 mg/l $CaCO_3$.	99, 98	

Conclusion: IPNV is sensitive to treatment with ozone. Based on these figures the dose needed for a 3 log reduction is unknown.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	ozone	15 sec	0,33 mg/l TRO	7,9	5	99,0 % reduction	Sea water, sterile filtered.	73	C T value: 5,0 mg*s/l, written in article.
	ozone	31 min	2,5 mg/l TRO	7,9	5	98,4 % reduction	Sea water, sterile filtered.	73	C T value: 4650 mg*s/l, written in article.

	ozone	14 min	6,7 mg/l TRO	7,9	5	98,0 % reduction	Sea water, sterile filtered.	73	C T value: 5628 mg*s/l, written in article.
	ozone	17 min	7,9 mg/l TRO	7,9	5	98,7 % reduction	Sea water, sterile filtered.	73	C T value: 8058 mg*s/, written in article. l

Conclusion: Based on this paper ISAV is sensitive to ozone but it will not be possible to obtain more than a 2 log reduction.

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10 µg purified virus	ozone	30 sec	0,1 mg/ml TRO		20	Not inactivated (0/800 larvae survived, control 238/800)	Virus added to 1 ml ozone treated sea water. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV. C T value: 3 mg*s/l
	ozone	1 min	0,2 mg/ml TRO			”Effective” (136/800 larvae survived, antigen ELISA negative, untreated eggs 0/800 survived, antigen ELISA positive)	Washihng of eggs from noda infected ovaries i sea water, followed after hatching.	9	Isolate: SJNNV C T value: 12 mg*s/l
10 µg purified virus	ozone	2,5 min	0,1 mg/ml TRO		20	”Effective” (334/800 larvae survived, antigen ELISA negativ, control 238/800)	Virus added to 1 ml ozone treated sea water. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV. C T value: 15 mg*s/l
10 µg purified virus	ozone	30 sec	0,5 mg/ml TRO		20	”Effective” (150/800 larvae survived, antigen ELISA negativ, control 238/800)	Virus added to 1 ml ozone treated sea water. Used for infection trial in day old striped jack larvae.	9	Isolate: SJNNV. C T value: 15 mg*s/l
	ozone	31,5 min	1,6 mg/l TRO	7,9	5	98,0 % reduction	Sea water, sterile filtered.	73	C T value: 3043 mg*s/l, written in article.

Conclusion: Nodavirus is sensitive towards ozone. One papers state that a dose of 12-15 mg*s/l is effective in prohibiting disease in striped jack larvae. The other paper states that when using a dose of 3000 mg*s/l only a 2 log reduction is obtainable.

Bacteria

Aeromons licquefaciens

Concentration	Disinfectant	Contact	Concentration	pH	Temp.	Result	Method	Reference	Comments

pathogen		time	disinfectant						
10^7/ml	ozone	> 6 min	0,1 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: > 36 mg*s/l
10^7/ml	ozone	3 min	0,15 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 27 mg*s/l
10^7/ml	ozone	2 min	0,2 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 24 mg*s/l
10^7/ml	ozone	3½ min	0,2 mg/l	7		"inactivated"	Distilled water, continous ozonation.	16	Read off a graph. C T value: 42 mg*s/l
10^8/ml	ozone	1 min	1 mg/l	7		app. 3 log reduction	Distilled water, ozonation stopped when bacteria added.	16	No further reduction during the next 4 min. Read off a graph.

Conclusion: Based on this paper a dose of 30 mg*s/l is capable of a 4 log reduction of *A. licquefaciens.*

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^3 cells/ml	ozone	10 min	0,01 mg/l residual	6,9	20	"inactivated"	Distilled water.. Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	C T value: 6 mg*s/l
10^3 cells/ml	ozone	30 sec	0,04 mg/l residual	6,9	20	"inactivated"	Distilled water.. Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	C T value: 1,2 mg*s/l
10^7 CFU/ml	ozone	1 min	0,065 mg/l/sec	7,8	7	3½ log reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Read off a graph.
10^7 CFU/ml	ozone		0,065 mg/l/sec	7,8	7	99,9 % reduction	Wastewater from aquaculture (15,7 ‰ salinity).	71	Reduction rate: 0,12/sec
10^7/ml	ozone	6 min	0,05 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 18 mg*s/l
10^7 CFU/ml	ozone		0,1 mg/l/sec	7,2	7	99,9 % reduction	PBS	71	Reduction rate: 0,32/sec
Unknown	ozone		0,1 mg/l residual			Not detectable	Test af laboratoriespildevand	10	A. salm is known to be part of the wastewater, but it has not been tested whether it was possible to re-isolate the bacteria before ozonation.
10^7/ml	ozone	1½ min	0,1 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 9 mg*s/l
10^7/ml	ozone	2½ min	0,1 mg/l	7		"inactivated"	Distilled water, continous ozonation.	16	Read off a graph. C T value: 15 mg*s/l

3 x 10^6 CFU/ml	ozone	180 sec	0,15 mg/l		9-12	4 log reduction	Sea water, salinity 32 ‰, autoclaved.	70	C T value: 27 mg*s/l
3 x 10^6 CFU/ml	ozone	120 sec	0,15 mg/l		9-12	4 log reduction	Brackish water, salinity 15 ‰, autoclaved.	70	Read off a graph. C T value: 18 mg*s/l
3 x 10^6 CFU/ml	ozone	60 sec	0,20 mg/l		9-12	4 log reduction	Lake water, autoclaved.	70	Read off a graph. C T value: 12 mg*s/l
10^8/ml	ozone	1 min	1 mg/l	7		”inactivated”	Distilled water, ozonation stopped when bacteria added.	16	Read off a graph.
10^3 cells/ml	ozone	30 min	20 mg/h/l	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	
10^3 cells/ml	ozone	5 min	90 mg/h/l	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	
10^3 cells/ml	ozone	15 min	90 mg/h/l	6,9	20	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	

Conclusion: *A. salmonicida* is sensitive to treatment with ozone. Based on these figures the dose needed for a 4 log reduction is 10-30 mg*s/l.

Enterococcus seriolicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,5}$ cfu/ml	ozone	6 min	0,018 mg/l TRO			Stable	Sea water, sterile filtered.	92	Read off a graph. C T value: 6,48 mg*s/l
$10^{6,5}$ cfu/ml	ozone	4 min	0,096 mg/l TRO			”inactivated”	Sea water, sterile filtered.	92	Read off a graph. C T value: 23,04 mg*s/l
$10^{6,5}$ cfu/ml	ozone	1 min	0,536 mg/l TRO			”inactivated”	Sea water, sterile filtered.	92	Read off a graph. C T value: 32,16 mg*s/l
	ozone	1 min	0,393 mg/l TRO			6 log reduction	Estimated based on Chick-Watson parametre.	92	C T value: 23,58 mg*s/l

Natural flora (heterotrophic bacteria)

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
116 ± 25 cfu/ml	ozone	8,3 min	0,21 mg/l	7,5	14,3	1,35 log reduction	fish farm, recirculation	87	C T value: 105 mg*s/l
4,7 x 10^4 cfu/ml	ozone	1 min	0,5 mg/l TRO			4 log reduction	flowrate 2,0 m^3	56	Natural flora in wastewater from hathing facility.

									C T value: 30 mg*s/l
$10^{5,5}$ cfu/ml	ozone	3 min	0,773 mg/l TRO			”inactivated”	havvand	92	Read off a graph. C T value: 140 mg*s/l
Unknown	ozone		1,0 mg/l residual			Survival		10	
$10^{5,5}$ cfu/ml	ozone	< 1 min	1,933 mg/l TRO			”inactivated”	havvand	92	Read off a graph. C T value: < 120 mg*s/l

Pasteurella piscicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^6 cfu/ml	ozone	6 min	0,018 mg/l TRO			Stable	Sea water, sterile filtered.	92	Read off a graph. C T value: 6,5 mg*s/l
	ozone	1 min	0,165 mg/l TRO			6 log reduction	Estimated based on Chick-Watson parametre.	92	C T value: 10 mg*s/l
10^6 cfu/ml	ozone	1 min	0,370 mg/l TRO			”inactivated”	Sea water, sterile filtered.	92	Read off a graph. C T value: 22 mg*s/l

Pseudomonas flourescens

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7/ml	ozone	4½ min	0,1 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 27 mg*s/l
10^7/ml	ozone	2½ min	0,15 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 22,5 mg*s/l
10^7/ml	ozone	2 min	0,2 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 24 mg*s/l
10^7/ml	ozone	2½ min	0,15 mg/l	7		”inactivated”	Distilled water, continous ozonation.	16	Read off a graph. C T value: 22,5 mg*s/l
108/ml	ozone	1 min	1 mg/l	7		app. 3 log reduction	Distilled water, ozonation stopped when bacteria added.	16	No further reduction during the next 4 min. Read off a graph.

Conclusion: *P. flourescens* is sensitive to treatment with ozone. Based on these figures the dose needed for a 4 log reduction is 20-30 mg*s/l.

Renibacterium salmoninarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
unknown	ozone		0,1 mg/l residual			Not detectable	Test af laboratoriespildevand	10	R. salm is known to be part of the wastewater, but it has not been tested whether it was possible to re-isolate the bacteria before ozonation.

Vibrio anguillarum

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^6 cfu/ml	ozone	6 min	0,018 mg/l TRO			Stable	Sea water, sterile filtered.	92	Read off a graph. C T value: 6,5 mg*s/l
	ozone	1 min	0,029 mg/l TRO			6 log reduction	Estimated based on Chick-Watson parametre.	92	C T value: 1,8 mg*s/l
unknown	ozone		0,1 mg/l residual			Not detectable	Test af laboratoriespildevand	10	V. ang is known to be part of the wastewater, but it has not been tested whether it was possible to re-isolate the bacteria before ozonation.
3 x 10^8 CFU/ml	ozone	60 sec	0,15 mg/l		9-12	app. 5 log reduction	Sea water, salinity 32 ‰, autoclaved.	70	Read off a graph. C T value: 9 mg*s/l
3 x 10^8 CFU/ml	ozone	60 sec	0,15 mg/l		9-12	app. 6 log reduction	Brackish water, salinity 15 ‰, autoclaved.	70	Read off a graph. C T value: 9 mg*s/l
10^6 cfu/ml	ozone	1½ min	0,196 mg/l TRO			”inactivated”	Sea water, sterile filtered.	92	Read off a graph. C T value: 18 mg*s/l
3 x 10^8 CFU/ml	ozone	60 sec	0,20 mg/l		9-12	app. 5 log reduction	Lake water, autoclaved.	70	Read off a graph. C T value: 12 mg*s/l

Conclusion: *V. anguillarum* is sensitive to treatment with ozone. Based on these figures the dose needed for a 5-6 log reduction is 10-20 mg*s/l. The results indicate that there is a minimum TRO needed in order for the ozone to inactivate the microorganism. When *V. anguillarum* was treated with

0,018 mg/l TRO (6m5 mg*s/l) for 6 min the titer was stable, whereas treatment using 0,029 mg/l TRO (1,8 mg*s/l) for 1 min the bacteria was inactivated.

Vibrio salmoicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
3 x 10^6 CFU/ml	ozone	60 sec	0,20 mg/l		9-12	4 log reduction	Lake water, autoclaved.	70	C T value: 12 mg*s/l
3 x 10^6 CFU/ml	ozone	120 sec	0,15 mg/l		9-12	4 log reduction	Sea water, salinity 32 ‰, autoclaved.	70	C T value: 18 mg*s/l
3 x 10^6 CFU/ml	ozone	180 sec	0,15 mg/l		9-12	4 log reduction	Brackish water, salinity 15 ‰, autoclaved.	70	C T value: 27 mg*s/l

Conclusion: *V. salmonicida* is sensitive to treatment with ozone. Based on these figures the dose needed for a 4 log reduction is 10-30 mg*s/l.

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^3 cells/ml	ozone	30 sec	0,01 mg/l residual	6,9	20	”inactivated”	Distilled water.. Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	C T value: 0,3 mg*s/l
10^7/ml	ozone	5½ min	0,05 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 16,5 mg*s/l
10^7/ml	ozone	4 min	0,1 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 24 mg*s/l
10^7/ml	ozone	2½ min	0,15 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 22,5 mg*s/l
3 x 10^9 CFU/ml	ozone	30 sec	0,15 mg/l		9-12	app. 6 log reduction	Sea water, salinity 32 ‰, autoclaved.	70	Read off a graph. C T value: 4,5 mg*s/l
3 x 10^9 CFU/ml	ozone	60 sec	0,15 mg/l		9-12	app. 7 log reduction	Brackish water, salinity 15 ‰, autoclaved.	70	Read off a graph. C T value: 9 mg*s/l
10^7/ml	ozone	1½ min	0,2 mg/l	7		app. 4 log reduction	Distilled water, continous ozonation.	16	Read off a graph. C T value: 18 mg*s/l
10^7/ml	ozone	1½ min	0,2 mg/l	7		”inactivated”	Distilled water, continous ozonation.	16	Read off a graph. C T value: 18 mg*s/l
3 x 10^9 CFU/ml	ozone	60 sec	0,20 mg/l		9-12	app. 7 log reduction	Lake water, autoclaved.	70	Read off a graph. C T value: 12 mg*s/l
10^8/ml	ozone	1 min	1 mg/l	7		app. 3½ log reduction	Distilled water, ozonation stopped when bacteria added.	16	No further reduction during the next 4 min. Read off a graph.
10^3 cells/ml	ozone	25 min	20 mg/h/l	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$ Samples tested after ½,	98, 97	

							1, 2, 5, 10, 20 and 30 min.		
10^3 cells/ml	ozone	25 min	20 mg/h/l	6,9	20	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	
10^3 cells/ml	ozone	10 min	90 mg/h/l	8,2	20	”inactivated”	Hard lake water., 120 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	
10^3 cells/ml	ozone	10 min	90 mg/h/l	6,9	20	”inactivated”	Soft lake water., 30 mg/l $CaCO_3$ Samples tested after ½, 1, 2, 5, 10, 20 and 30 min.	98, 97	

Conclusion: *Y. ruckeri* is sensitive to treatment with ozone. Based on these figures the dose needed for a ≥ 4 log reduction is 10-30 mg*s/l.

Other oxidising disinfectants

Virus

IPNV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,08\text{-}7,41}$ $TCID_{50}$/ml	pentakalium bis (peroxymonsulfate) bis (sulfat)	30 min	0,5 % (v/v)		4	> 4 log reduction	Tested at 0,1, 0,2 and 0,5%. Hard water, high organic load.	49	Contains > 10% available oxygen.
	peracetic acid (divosan forte)	39 days	0,5%		4	Survival	Mixing of virus and fish silage.	91	Type Sp. Titer reduction 2,75. Titer day 39 without Divosan Forte: 5,45, with DF 2,70
	peracetic acid (divosan forte)	16 days	5%		4	> 2,45 reduction	Mixing of virus and fish silage.	91	Type Sp. Titer reduction > 2,45. Titer day 16 without Divosan Forte: 5,45, with DF < 3.00
$10^{6,58\text{-}6,74}$ $TCID_{50}$/ml	peracetic acid/hydrogen peroxid	30 min	0,276% (v/v)		4	> 4 log reduction	Tested at 0,16, 0,276 and 1,6%. Hard water, high organic load.	49	
4 x 10^6 pfu/ml	VirkonS in fish silage treated with formic acid and	30 min	1/100 w/v	?	?	”inactivated”	Mixing of virus and fish silage.	90, 91	Startdosis: 4x10^6, slutdosis <400

	propionic acid								
$10^{5,00\text{-}6,23}$ $TCID_{50}$/ml	Hydrogenperoxid/acetic acid/peracetic acid	30 min	1,0%		4	> 4 log reduction	Sea water. Contact time 15 and 30 min. Koncentration: 0,5, 1,0, 1,5 and 2,0%. 1% BSA + 1% yeast extract.	18	IPNV isolat N1. Kick-Start2: H_20_2 20%, organic acids > 10%, peracetic acid 5%, surfactant, stabilizing and complex inducing agents.

ISAV

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
5,0 log10 ffu/ml	peracetic acid/H_2O_2/acetic acid	5 min	1:80		4	> 4,7 log reduction	Hard water, 342 ppm total hardness, no addition of serum.	89	Proxitane
5,0 log10 ffu/ml	peracetic acid/H_2O_2/acetic acid	5 min	1:80		4	> 4,7 log reduction	Hard water, 342 ppm total hardness, addition of serum.	89	Proxitane

Nodavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	5 min	1:125 w/v		15	3,8 log reduction	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	30 min	1:125 w/v		15	3,3 log reduction	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	5 min	1:500 w/v		15	Stable	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	30 min	1:500 w/v		15	3,3 log reduction	Distilled water.. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	5 min	1:125 w/v		15	2,8 log reduktoin	HBSS+calf serum. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	30 min	1:125 w/v		15	2,8 log reduktoin	HBSS+calf serum. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus
$10^{6,375}$ $TCID_{50}$/ml	Virkon (peroxygen)	30 min	1:500 w/v		15	Stable	HBSS+calf serum. Tested after 5, 15 and 30 min.	30	Isolate: sea bass nodavirus

Oncorhynchus masou virus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	kaliumpermanganat	30 sec	32		0	”inactivated”		35	
	kaliumpermanganat	30 sec	16		15	”inactivated”		35	
	kaliumpermanganat	30 sec	16		25	”inactivated”		35	

Ranavirus

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
1 x 10^7 PFU/ml	VirkonS	1 min	1%		22	”inactivated”		14	Isolate from American bullfrog

Aeromonas salmonicida

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pentakalium bis (peroxymonsulfate) bis (sulfat)	30 min	0,5 % (w/v)		4	> 5 log reduction	Tested at 0,01, 0,05, 0,1, 0,2, 0,5 and 1%. Hard water, high organic load.	49	Contains > 10% available oxygen.
	peracetic acid/hydrogen peroxid	30 min	0,1% (w/v)		4	> 5 log reduction	Tested at 0,05, 0,1, 0,2, 0,33 and 0,5%. Hard water, high organic load..	49	

Carnobacterium piscicola

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pentakalium bis (peroxymonsulfate) bis (sulfat)	30 min	0,5 - 1% (w/v)		4	> 5 log reduction	Tested at 0,01, 0,05, 0,1, 0,2, 0,5 and 1%. Hard water, high organic load.	49	Contains > 10% available oxygen.
	peracetic acid/hydrogen peroxid	30 min	0,2 % (v/v)		4	> 5 log reduction	Tested at 0,05, 0,1, 0,2, 0,33 and 0,5%. Hard water, high organic load..	49	

Edwardsiella tarda

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	H_2O_2	20 min	1600 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	H_2O_2	60 min	1600 ppm		20	> 5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Lactococcus garviae

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pentakalium bis (peroxymonsulfate) bis (sulfat)	30 min	0,5 - >1% (w/v)		4	> 5 log reduction	Tested at 0,01, 0,05, 0,1, 0,2, 0,5 and 1%. Hard water, high organic load.	49	Contains > 10% available oxygen.
	peracetic acid/hydrogen peroxid	30 min	0,2 - 0,3 % (v/v)		4	> 5 log reduction	Tested at 0,05, 0,1, 0,2, 0,33 and 0,5%. Hard water, high organic load..	49	

Streptococcus sp

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	H_2O_2	20 min	3200 ppm		20	5 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	H_2O_2	60 min	1600 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Vibrio sp.

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
10^7 CFU/ml	H_2O_2	20 min	1600 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm). Growht at 20°C and counting already after 24 hours.	58	The actual concentration of disinfectant probably only half of stated in article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.
10^7 CFU/ml	H_2O_2	60 min	800 ppm		20	4 log reduction	Dilution 1:1 of bacteria and disinfectant (25, 50, 100, 200, 400, 800, 1600, 3200 ppm).	58	The actual concentration of disinfectant probably only half of stated in

							Growht at 20°C and counting already after 24 hours.		article table. Generally speaking the neccessary concentration for disinfection in this paper is much higher than published in other papers.

Yersinia ruckeri

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	pentakalium bis (peroxymonsulfate) bis (sulfat)	30 min	0,5 % (w/v)		4	> 5 log reduction	Tested at 0,01, 0,05, 0,1, 0,2, 0,5 and 1%. Hard water, high organic load.	49	Contains > 10% available oxygen.
	peracetic acid/hydrogen peroxid	30 min	0,2 % (v/v)		4	> 5 log reduction	Tested at 0,05, 0,1, 0,2, 0,33 and 0,5%. Hard water, high organic load..	49	

Parasites

Ichthybodo necator

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	Detarox (20% H_2O_2, 4-5% peracetic acid)					All dead	2 treatments of naturally infected trout.	45	

Ichthyophthirius multifiliis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	H2O2	10 hours	50 µl/l		20	Stable	In vitro - trophonts	66	
	perotan	10 hours	50 µl/l		20	Stable	In vitro - trophonts	66	perotan: H_2O_2 + acetic acid
	perotan	10 hours	100 µl/l		20	Døde	In vitro - trophonts	66	perotan: H_2O_2 + acetic

									acid
	VirkonS	10 hours	50 µl/l		20	Stable	In vitro - trophonts	66	

Myxosoma cerebralis

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
	H_2O_2	10 min	8,5 %		room temp.	Survival	in vitro. Triactinomyxon spores	96	
	H_2O_2	10 min	10,2%		room temp.	All dead	in vitro. Triactinomyxon spores	96	
	$KMnO_4$	14 days	1%		22	Survival	In vitro - spores	42	

Trichodina jadranica

Concentration pathogen	Disinfectant	Contact time	Concentration disinfectant	pH	Temp.	Result	Method	Reference	Comments
2,4	Detarox (20% H2O2, 4-5% peracetic acid)	24 hours	45 ppm		25	Survival (0,4)	In vivo, ål	75	Catergorization (category/number of parasites on ell): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000
2,7	H2O2	4 hours	1000 ppm		25	Survival (2,3)	In vivo, ål	75	Catergorization (category/number of parasites on ell): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000
2,0	kaliumpermanganat	24 hours	20 ppm		25	All dead	In vivo, ål	75	Catergorization (category/number of parasites on ell): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000
2,6	VirkonPF	24 hours	20 ppm		25	All dead	In vivo, ål	75	Catergorization (category/number of parasites on ell): 0/0, 1/1-10, 2/11-100, 3/100-1000, 4/>1000

Reference List

1. Anon. (2009): Gyrodactylosis (*Gyrodactylus salaris*). *OIE Manual of Diagnostic Tests for Aquatic Animals* .

2. Anon. Disinfectants. Chlorine. http://www.lenntech.com/processes/disinfection/chemical/disinfectants-chlorine.htm. Assessed 17-03-2011.

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

4. Ahne W (1982): Vergleichende Untersuchung über die Stabilität von vier fischpathogenen Viren (VHSV, PFR, SVCV, IPNV). *Zentralblatt für Veterinarmedizin* **29**(6): 457-476.

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

6. Ahne W and Held C (1980): Untersuchungen über die viruzide Wirkung von Actomar K30* auf fischpathogene Viren. *Zeitschrift für alle Gebiete der Veterinärmedizin* **35**(5): 308-318.

7. Amend DF (1975): Detection and transmission of infectious haematopoietic necrosis virus in rainbow trout. *Journal of Wildlife Diseases* **11**(4): 471-478.

8. Amend DF and Pietsch JP (1972): Virucidal activity of two iodophors to salmonid viruses. *Journal of the Fisheries Research Board of Canada* **29**(1): 61-65.

9. 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**(1): 15-22.

10. Austin B (1983): Effectiveness of ozone for disinfection of laboratory effluent. *FEMS Microbiology Letters* **19**: 211-214.

11. Batts WN, Landolt ML, and Winton JR (1991): Inactivation of infectious hematopoietic necrosis virus by low levels of iodine. *Applied and Environmental Microbiology* **57**(5): 1379-1385.

12. Bootland LM, Dobos P, and Stevenson RMW (1991): The IPNV Carrier State and Demonstration of Vertical Transmission in Experimentally Infected Brook Trout. *Diseases of Aquatic Organisms* **10**(1): 13-21.

13. Brudeseth BE, Skall HF, and Evensen Ø (2008): Differences in virulence of marine and freshwater isolates of viral hemorrhagic septicemia virus in vivo correlate with in vitro ability to infect gill epithelial cells and macrophages of rainbow trout (*Oncorhynchus mykiss*). *Journal of Virology* **82**(21): 10359-10365.

14. Bryan LK, Baldwin CA, Gray MJ, and Miller DL (2009): Efficacy of select disinfectants at inactivating Ranavirus. *Diseases of Aquatic Organisms* **84**(2): 89-94.

15. Bullock G and Stuckey HM (1977): Ultraviolet treatment of water for destruction of five gram negative bacteria pathogenic to fishes. *Journal of the Fisheries Research Board of Canada* **34**(8): 1244-1249.

16. Colberg PJ and Lingg AJ (1978): Effect of ozonation on microbial fish pathogens, ammonia, nitrate, nitrite, and BOD in simulated reuse hatchery water. *Journal of the Fisheries Research Board of Canada* **35**(10): 1290-1296.

17. 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.

18. Devold M and Nylund A (2002) Disinfecting effect of Kick-Start2/D50 against IPN virus in seawater and in standardized hard water. Protevs AS, Thormølensgate 55, 5008 Bergen, Norway.

19. Dixon PF, Joiner C, and Way K (2007) P-07: First occurrence of viral haemorrhagic septicaemia virus in rainbow trout in the United Kingdom: studies on the virulence of the virus for rainbow trout and brown trout. *13th international conference of the EAFP* **Abstract book**, 144.

20. Dixon PF, Smail DA, Algoet M, Hastings T, Bayley A, Byrne H, Dodge M, Garden A, Joiner C, Roberts E, and Thompson F (2003) Inactivation of fish pathogenic viruses and bacteria for waste disposal purposes: laboratory and field studies. *12th International Conference of the EAFP* [Abstract O-030], 35.

21. Dorson M and Torchy C (1981): The influence of fish age and water temperature on mortalities of rainbow trout, *Salmo gairdneri* Richardson, caused by a European strain of infectious pancreatic necrosis virus. *Journal of Fish Diseases* **4**(3): 213-221.

22. Eggset G and Sommer A-I (1990) SETAX - effect på fiskepatogene bakterier og virus. Uttesting av betingelser for dreping. **R 0404**, 1-11. FORUT (Forskningsstiftelsen ved universitetet i Tromsø).

23. Elliot DG and Amend DF (1978): Efficacy of certain disinfectants against infectious pancreatic necrosis virus. *Journal of Fish Biology* **12**(4): 277-286.

24. Eskildsen UE and Jørgensen PEV (1974): Jodophorer som desinfektionsmiddel i dambrugene. *Ferskvandsfiskeribladet* **72**(12): 1-3.

25. Evelyn TPT, Ketcheson JE, 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**(3): 173-182.

26. Evensen Ø, Meier W, Wahli T, Olesen NJ, Jørgensen PEV, and Håstein T (1994): Comparison of immunohistochemistry and virus cultivation for detection of viral haemorrhagic septicaemia virus in experimentally infected rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **20**(2): 101-109.

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

28. Fløgstad H, Torgersen Y, Schei I, and Røttereng PJ (1991) Desinfeksjon af blodvann fra fiskeslakterier. **STF60 A91096**, 101 pp. Trondheim, SINTEF.

29. Fregeneda-Grandes JM, Skall HF, and Olesen NJ (2009): Antibody response of rainbow trout with single or double infections involving viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *Diseases of Aquatic Organisms* **83**(1): 23-29.

30. 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**(1-2): 13-24.

31. Goldes SA and MEAD SL (1995): Efficacy of Iodophor Disinfection against Egg Surface-Associated Infectious Hematopoietic Necrosis Virus. *The Progressive Fish-Culturist* **57**(1): 26-29.

32. Gosting LH and Gould RW (1981): Thermal inactivation of infectious hematopoietic necrosis and infectious pancreatic necrosis viruses. *Applied and Environmental Microbiology* **41**(4): 1081-1082.

33. Graham DA, Staples C, Wilson CJ, Jewhurst H, Cherry K, Gordon A, and Rowley HM (2007): Biophysical properties of salmonid alphaviruses: influence of temperature and pH on virus survival. *Journal of Fish Diseases* **30**(9): 533-543.

34. Gratzek JB, Gilbert JP, Lohr AL, Shotts Jr EB, and Brown J (1983): Ultraviolet light control of *Ichthyophthirius multifiliis* Fouquet in a closed fish culture recirculation system. *Journal of Fish Diseases* **6**(2): 145-153.

35. Hatori S, Motonishi A, Nishizawa T, and Yoshimizu M (2003): Virucidal effect of disinfectants against *Oncorhynchus masou* virus (OMV). *Fish Pathology - Gyobyo Kenkyu* **38**(4): 185-187.

36. Hedrick RP, Petri B, McDowell TS, Mukkatira K, and Sealey LJ (2007): Evaluation of a range of doses of ultraviolet irradiation to inactivate waterborne actinospore stages of *Myxobolus cerebralis*. *Diseases of Aquatic Organisms* **74**(2): 113-118.

37. Hedrick RP, McDowell TS, Mukkatira K, MacConnell E, and Petri B (2008): Effects of freezing, drying, ultraviolet irradiation, chlorine, and quaternary ammonium treatments on the infectivity of myxospores of *Myxobolus cerebralis* for *Tubifex tubifex*. *Journal of Aquatic Animal Health* **20**(2): 116-125.

38. Hershberger PK, Kocan RM, Elder NE, Meyers TR, and Winton JR (1999): Epizootiology of viral hemorrhagic septicemia virus in Pacific herring from the spawn-on-kelp fishery in Prince William Sound, Alaska, USA. *Diseases of Aquatic Organisms* **37**(1): 23-31.

39. Hirvelä-Koski V (2004): Renibacterium salmoninarum: effect of hypochlorite treatment, and survival in water. *Diseases of Aquatic Organisms* **59**(1): 27-33.

40. Hoffman GL (1974): Disinfection of Contaminated Water by Ultraviolet Irradiation, with Emphasis on Whirling Disease (Myxosoma cerebralis) and Its Effect on Fish. *Transactions of the American Fisheries Society* **103**: 541-550.

41. Hoffman GL (1975): Whirling disease (Myxosoma cerebralis): control with ultraviolet irradiation and effect on fish. *Journal of Wildlife Diseases* **11**(4): 505-507.

42. Hoffman GL, Sr. and Hoffman GL, Jr. (1972): Studies on the control of whirling disease (*Myxosoma cerebralis*).: I. The effects of chemicals on spores in vitro, and of calcium oxide as a disinfectant in simulated ponds. *Journal of Wildlife Diseases* **8**(1): 49-53.

43. Hoffman GL and Markiw ME (1977): Control of whirling disease (*Myxosoma cerebralis*): use of methylene blue staining as a possible indicator of effect of heat on spores. *Journal of Fish Biology* **10**(2): 181-183.

44. Hoffman GL and O'Grodnick JJ (1977): Control of whirling disease (*Myxosoma cerebralis*): effects of drying, and disinfection with hydrated lime or chlorine. *Journal of Fish Biology* **10**(2): 175-179.

45. Holten K, Skjelstad B, Aunsmo A, and Skjelstad HR (2003): Effect of Detarox(R)AP against infection with Ichthyobodo necator. *Norsk Veterinærtidsskrift* **115**(11): 719-723.

46. Huber P, Petri B, Allen S, and Lumsden JS (2010): Viral haemorrhagic septicaemia virus IVb inactivation by ultraviolet light, and storage viability at 4 and -20 °C. *Journal of Fish Diseases* **33**(4): 377-380.

47. Husby A (2003) Hygieniserende effekt av Gellyfeed-prosessen. **4502/107**, 15 pp. Trondheim, Stiftelsen Rubin.

48. Jacobsen P, Liltved H, and Efraimsen H (1989): Disinfection of effluent from fish slaughteries. *Aquacultural Engineering* **8**(3): 209-216.

49. Jeffreys DW, Joiner CL, Bagwell NJ, Reese RA, Husby A, and Dixon PF (2009): Development of bactericidal and virucidal testing standards for aquaculture disinfectants. *Aquaculture* **286**(3-4): 190-197.

50. Jørgensen PEV and Olesen NJ: Indflydelse af vandtemperatur og smitteintensitet på sygdommens forløb, in Jørgensen PEV and Olesen NJ (Eds): *Bekæmpelse af egtvedsyge (VHS) hos regnbueørreder*, Fællesudvalget for det danske ørrederhverv, 1983, pp 16-21.
Ref Type: Book Chapter

51. Jørgensen PEV: Untersuchungen über das Egtved-virus: Seine Stabilität und Identifizierung, in Liebmann H (Ed): *Die hämorrhagische Virusseptikämie (HVS) der Forellen*. München und Wien, 1970, pp 60-71.
Ref Type: Book Chapter

52. Jørgensen PEV (1973): Inactivation of IPN and Egtved virus. *Rivista Italiana di Piscicoltura e Ittiopatologia* **4**(VIII): 107-108.

53. Jorquera MA, Valencia G, Eguchi M, Katayose M, and Riquelme C (2002): Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment. *Aquaculture* **207**(3-4): 213-224.

54. Kasai H, Ishikawa A, Hori Y, Watanabe K, and Yoshimizu M (2000): Disinfectant effects of electrolyzed salt water on fish pathogenic bacteria and viruses. *Nippon Suisan Gakkaishi* **66**(6): 1020-1025.

55. Kasai H, Muto Y, and Yoshimizu M (2005): Virucidal effets of ultraviolet, heat treatment and disinfectants against koi herpesvirus (KHV). *Fish Pathology - Gyobyo Kenkyu* **40**(3): 137-138.

56. Kasai H, Watanabe K, and Yoshimizu M (2001): Bactericidal effect of continuous flow electrolyzer on hatchery waste-seawater. *Nippon Suisan Gakkaishi* **67**(2): 222-225.

57. Kasai H, Watanabe K, and Yoshimizu M (2001): Disinfectant effects of hypochlorite produced by batch electrolytic system on fish pathogenic bacteria and virus. *Suisan Zoshoku* **49**(2): 237-241.

58. Kim S-R, Park K-H, Kim D, Jung S-J, Kang S-Y, and Oh M-J (2008): Antimicrobial effects of chemical disinfectants on fish pathogenic bacteria. *Food Science and Biotechnology* **17**(5): 971-975.

59. Kimura T, Yoshimizu M, Tajima K, and Ezura Y (1980): Disinfection of hatchery water supply by ultraviolet (U. V.) irradiation - II. U.V. susceptibility of some fish pathogenic fungi. *Fish Pathology - Gyobyo Kenkyu* **14**(3): 133-137.

60. Kimura T, Yoshimizu M, Tajima K, Ezura Y, and Sakai M (1976): Disinfection of hatchery water supply by UV irradiation part I. Susceptibility of some fish pathogenic bacterium and microorganisms inhabiting pond waters. *Nippon Suisan Gakkaishi* **42**(2): 207-211.

61. Kiryu I, Sakai T, Kurita J, and Iida T (2007): Virucidal effect of disinfectants on spring viremia of carp virus. *Fish Pathology - Gyobyo Kenkyu* **42**(2): 111-113.

62. Kocan R, Bradley M, Elder N, Meyers TR, Batts WN, and Winton JR (1997): North American strain of viral hemorrhagic septicemia virus is highly pathogenic for laboratory-reared pacific herring. *Journal of Aquatic Animal Health* **9**(4): 279-290.

63. Kocan RM, Hershberger PK, Elder NE, and Winton JR (2001): Epidemiology of viral hemorrhagic septicemia among juvenile pacific herring and Pacific sand lances in Puget Sound, Washington. *Journal of Aquatic Animal Health* **13**(2): 77-85.

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

65. 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 - Gyobyo Kenkyu* **37**(4): 175-181.

66. Lahnsteiner F and Weismann T (2007): Treatment of Ichthyophthiriasis in Rainbow Trout and Common Carp with Common and Alternative Therapeutics. *Journal of Aquatic Animal Health* **19**(3): 186-194.

67. 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**(4): 295-310.

68. Liltved H (2000) Disinfection of water in aquaculture: factores influencing the physical and chemical inactivation of microorganisms. The Norwegian College of Fishery Scinece, University of Tromsø, Norway.

69. Liltved H and Cripps SJ (1999): Removal of particle-associated bacteria by prefiltration and ultraviolet irradiation. *Aquaculture Research* **30**(6): 445-450.

70. 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. *Aquacultural Engineering* **14**(2): 107-122.

71. Liltved H and Landfald B (1995): Use of alternative disinfectants, individually and in combination, in aquacultural wastewater treatment. *Aquaculture Research* **26**(8): 567-576.

72. Liltved H and Landfald B (1996): Influence of liquid holding recovery and photoreactivation on survival of ultraviolet-irradiated fish pathogenic bacteria. *Water Research* **30**(5): 1109-1114.

73. Liltved H, Vogelsang C, Modahl I, and Dannevig BH (2006): High resistance of fish pathogenic viruses to UV irradiation and ozonated seawater. *Aquacultural Engineering* **34**(2): 72-82.

74. MacKelvie RM and Desautels D (1975): Fish viruses - suvival and inactivation of infectious pancreatic necrosis virus. *Journal of the Fisheries Research Board of Canada* **32**(8): 1267-1273.

75. Madsen HCK, Buchmann K, and Mellergaard S (2000): Treatment of trichodiniasis in eel (*Anguilla anguilla*) reared in recirculation systems in Denmark: alternatives to formaldehyde. *Aquaculture* **186**(3-4): 221-231.

76. Maisse G, Dorson M, and Torchy C (1980): Inactivation de deux virus pathogénes pour les salmonides (virus de la nécrose Pancréatique infectieuse et de la septicémie hémorragique virale) par les rayons ultraviolets. *Bulletin Français de Pisciculture* **278**(3): 34-40.

77. Mulcahy D, Jenes CK, and Pascho R (1984): Appearance and quantification of infectious hematopoietic necrosis virus in female sockeye salmon (*Oncorhynchus nerka*) during their spawning migration. *Archives of Virology* **80**(2-3): 171-181.

78. Mulcahy D, Pascho RJ, and Jenes CK (1983): Detection of infectious haematopoietic necrosis virus in river water and demonstration of waterborne transmission. *Journal of Fish Diseases* **6**(4): 321-330.

79. Nelson RT, McLoughlin MF, Rowley HM, Platten MA, and McCormick JI (1995): Isolation of a toga-like virus from farmed Atlantic salmon Salmo salar with pancreas disease. *Diseases of Aquatic Organisms* **22**(1): 25-32.

80. Olstad K, Cable J, Robertsen G, and Bakke TA (2006): Unpredicted transmission strategy of *Gyrodactylus salaris* (Monogenea: Gyrodactylidae): survival ind infectivity of parasites on dead hosts. *Parasitology* **133**: 33-41.

81. 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.

82. Pascho RJ, Landolt ML, and Ongerth JE (1995): Inactivation of Renibacterium salmoninarum by free chlorine. *Aquaculture* **131**(3-4): 165-175.

83. 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**(9): 1360-1364.

84. 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**(9): 1359-1361.

85. Sako H, Ishida N, Maeno Y, and Sorimachi M (1988): Bactericidal activities of five disinfectants on *Aeromonas salmonicida*, *Vibrio anguillarum* and *V. ordalii*. *Fish Pathology - Gyobyo Kenkyu* **23**(4): 219-229.

86. 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.

87. Sharrer MJ and Summerfelt ST (2007): Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. *Aquacultural Engineering* **37**(2): 180-191.

88. Sharrer MJ, Summerfelt ST, Bullock GL, Gleason LE, and Taeuber J (2005): Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. *Aquacultural Engineering* **33**(2): 135-149.

89. Smail DA, Grant R, Simpson D, Bain N, and Hastings TS (2004): Disinfectants against cultured Infectious Salmon Anaemia (ISA) virus: the virucidal effect of three iodophors, chloramine T, chlorine dioxide and peracetic acid/hydrogen peroxide/acetic acid mixture. *Aquaculture* **240**(1-4): 29-38.

90. Smail DA, Huntly PJ, and Munro ALS (1990) Fate of four fish pathogens after exposure to an ensiling mixture containing fish farm mortalities. **CM 1990/F:50 Mariculture Committee**. International Council for the Exploration of the Sea.

91. 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.

92. Sugita H, Asai T, Hayashi K, Mitsuya T, Amanuma K, 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**(12): 4072-4075.

93. Tabandeh MR and Akhlaghi M (2007): Efficacy of conventional disinfectants on isolated Streptococcus iniae from diseased rainbow trout in laboratory and culture tank conditions. *Iranian Scientific Fisheries Journal* **16**(3): 29-38.

94. Torgersen Y (1998) Annex 5 Physical and chemical inactivation o fthe infectious salmon anaemia (ISA) virus. Håstein, T. Workshop on ISA. *Workshop on ISA.New Brunswick, St Andrews* , 44-53.

95. Tuttle-Lau MT, Phillips KA, and Gaikowski MP (2010) Evaluation of the efficacy of iodophor disinfection of walleye and northern pike eggs to eliminate viral hemorrhagic septicemia virus. *U.S.Geological Survey Fact Sheet* **2009-3107**, 1-4.

96. Wagner EJ, Smith M, Arndt R, and Roberts DW (2003): Physical and chemical effects on viability of the *Myxobolus cerebralis* triactinomyxon. *Diseases of Aquatic Organisms* **53**(2): 133-142.

97. Wedemeyer GA and Nelson NC (1977): Survival of two bacterial fish pathogens (*Aeromonas salmonicida* and the enteric redmouth bacterium) in ozonated, chlorinated, and untreated waters. *Journal of the Fisheries Research Board of Canada* **34**: 429-432.

98. Wedemeyer GA, Nelson NC, and Yasutake WT (1979): Potentials and limits for the use of ozone as a fish disease control agent. *Science and Engineering* **1**: 295-318.

99. Wedemeyer GA, Nelson NC, and Smith CA (1978): Survival of the salmonid viruses infectious haematopoietic 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.

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

101. Wolf K: Infectious pancreatic necrosis*, Fish viruses and fish viral diseases*, 1 ed. United States of America, Comstock Publishing Associates, 1988, pp 115-157.
Ref Type: Book Chapter

102. Yoshimizu M, Yoshinaka T, Hatori S, and Kasai H (2005): Survivability of fish pathogenic viruses in environmental water, and inactivation of fish disease. *Bulletin of Fisheries Research Agency* **Suppl. 2**: 47-54.

103. Yoshimizu M, Takizawa H, and Kimura T (1986): U.V. susceptibility of some fish pathogenic viruses. *Fish Pathology - Gyobyo Kenkyu* **21**(1): 47-52.

104. Yoshimizu M, Takizawa H, Sami M *et al.*: Disinfectant effects of ultraviolet erradiation on fish pathogens in hatchery water supply*,* in Hirano R and Hanyo H (Eds): *The Second Asian Fisheries Forum*. Manila, Philippines, Asian Fisheries Society, 1990, pp 643-646.
Ref Type: Book Chapter

Aquaculture 511 (2019) 634192

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Salmonid alphavirus subtype I isolated from clinically-diseased Atlantic salmon, *Salmo salar*, in freshwater culture

Sílvia Soares*, Svenja A. Elwenn, Maria Campbell, Patricia White, Nichola Still, Eann S. Munro

Marine Scotland Science, Marine Laboratory, Aberdeen, Scotland, UK

ARTICLE INFO

Keywords:
Pancreas disease
Salmonid alphavirus
Salmonid freshwater production
Disease outbreak

ABSTRACT

Pancreas disease (PD) is a disease of cultivated salmonids caused by salmonid alphavirus (SAV) and it is responsible for significant losses within the aquaculture industry, at the marine stage, in Ireland, Norway and Scotland.

In May 2018, an increased mortality of Atlantic salmon at a freshwater hatchery on the west coast of Scotland was reported to Marine Scotland Science, Fish Health Inspectorate. Site staff suspected an infectious pancreatic necrosis (IPN) outbreak, a condition frequently diagnosed at salmonid freshwater sites, but the RT-qPCR results were negative for IPNV and positive for salmonid alphavirus. This detection was subsequently confirmed as SAV subtype I by partial sequence analysis of the E2 gene. Histological examination revealed lesions in the pancreas, heart and skeletal muscle consistent with PD pathology.

This is the first published description of a confirmed clinical field case of SAV with pathology consistent with PD in freshwater Atlantic salmon.

1. Introduction

Pancreas disease (PD) is a viral disease of cultivated salmonids and it is responsible for significant economic losses to the aquaculture industry, at the marine stage, in Ireland, Norway and Scotland due to mortality percentage, carcass and fillet quality downgrade as well as treatment and management costs (Munro et al., 1984; Ruane et al., 2008; Aunsmo et al., 2010; Larsson et al., 2012; Lerfall et al., 2012; Taksdal et al., 2012; Jansen et al., 2017). In Scotland, Kilburn et al., 2012, reported from a single company database that PD accounted for the greatest loss in biomass of all infectious diseases in 2006 and 2007. In Ireland the estimated economical loss for the production period of 2003 and 2004 was €35 million of turnover and €12 million of profit (Ruane et al., 2008).

Pancreas disease was first diagnosed in the 1970s and first reported in Atlantic salmon, *Salmo salar*, by Munro et al., 1984. In rainbow trout, *Oncorhynchus mykiss*, the condition was first reported by Boucher and Baudin Laurencin, 1994, in France, whereby the authors named the condition sleeping disease (SD).

Pancreas disease typically affects Atlantic salmon in the seawater stage but it has also been reported in seawater-reared rainbow trout (Taksdal et al., 2007). Meanwhile SD has been reported mostly in freshwater-reared rainbow trout at all stages and occasionally in coho salmon, *Oncorhynchus kisutch*, (Boucher and Baudin Laurencin, 1994) and Alsatian charr, *Salvelinus fontinalis* x *Salvelinus alpinus* hybrid (Steinbauer et al., 2019).

Currently, there are no records of natural clinical PD outbreaks in Atlantic salmon in freshwater aquaculture in Ireland, Norway or Scotland (Cano et al., 2015; Jansen et al., 2017). However, different researchers (Boucher et al., 1995; McLoughlin et al., 1995; Desvignes et al., 2002; Cano et al., 2015) have been able to experimentally induce clinical PD in Atlantic salmon in the freshwater stages.

Clinical PD outbreaks are likely to occur at any time of the year, however, the majority of clinical outbreaks are observed in the summer and autumn months (Crockford et al., 1999; Rodger and Mitchell, 2007; Ruane et al., 2008). In outbreaks of clinical PD, fish demonstrate clinical signs of lethargy, inappetence, presence of high numbers of faecal casts in the cages and increased mortalities (McLoughlin and Graham, 2007; Jansen et al., 2017). The mortality may range from minimal percentages to up 63% (Crockford et al., 1999; Rodger and Mitchell, 2007; Graham et al., 2010; McCleary et al., 2014; Jansen et al., 2017). Internally, the pancreas, heart and skeletal muscle can be significantly affected with lesions (McLoughlin et al., 2002; McLoughlin and Graham, 2007).

The causative agent of PD is a single-stranded RNA virus, belonging to the genus *Alphavirus* of the family *Togaviridae* (Nelson et al., 1995), commonly named salmonid alphavirus (SAV). The virus was first isolated by Nelson et al., 1995 and since then, it has been characterized

* Corresponding author.
E-mail address: Silvia.Soares@gov.scot (S. Soares).

https://doi.org/10.1016/j.aquaculture.2019.06.006
Received 14 December 2018; Received in revised form 15 May 2019; Accepted 2 June 2019
Available online 04 June 2019

Table 1
Geographical distribution of the different subtypes of salmonid alphavirus (SAV) and fish species where SAV RNA was detected (modified from OIE, 2018, Boucher and Baudin Laurencin, 1994, Fringuelli et al., 2008, Snow et al., 2010, Graham et al., 2012, Bruno et al., 2014, McCleary et al., 2014, Ruane et al., 2018, Steinbauer et al., 2019).

SAV subtype	Host	Environment	Country
SAV I (PD*)	Atlantic salmon; common dab; plaice; rainbow trout	Sea water; fresh water	Ireland, UK (Northern Ireland, Scotland)
SAV 2 FW (SD**)	Rainbow trout; Atlantic salmon; coho salmon; Alsatian charr	Freshwater	France, Germany, Italy, Spain, Switzerland, Poland, UK (England, Scotland)
SAV 2 marine (PD*)	Atlantic salmon; common dab	Seawater	Norway, UK (Scotland)
SAV 3 (PD*)	Rainbow trout; Atlantic salmon	Seawater	Norway
SAV 4 (PD*)	Atlantic salmon	Sea water	Ireland, UK (Northern Ireland, Scotland)
SAV 5 (PD*)	Atlantic salmon; common dab; long rough dab; plaice	Sea water	UK (Scotland)
SAV 6 (PD*)	Atlantic salmon, ballan wrasse	Seawater	Ireland

* PD: pancreas disease.
** SD: sleeping disease. Note: SAV2 has been divided into two subgroups named freshwater variant (SAV2 FW) and marine variant (SAV2 MW), where infections with SAV2 FW in freshwater-reared rainbow trout are named SD and infections with SAV2 MW in seawater-reared Atlantic salmon are named PD (Jansen et al., 2017).

into 6 genotypes with distinct geographic distribution (Table 1).

Several studies have been performed to evaluate the likelihood of vertical transmission of SAV (Bratland and Nylund, 2009; Kongtorp et al., 2010; Jansen et al., 2017) but so far this association could not be determined. According to the Norwegian Scientific Committee for Food Safety, the risk of SAV being transmitted vertically is considered insignificant (Jansen et al., 2017). Conversely, the horizontal transmission of SAV is well studied and described (Kristoffersen et al., 2009; Jansen et al., 2010). Various subtypes of the virus have also been either isolated in cell culture or viral RNA detected by using RT-qPCR in other non-salmonid species (Table 1) such as common dab (*Limanda limanda*), plaice (*Pleuronectes platessa*), long rough dab (*Hippoglossoides platessoides*), ballan wrasse, *Labrus bergylta* (Snow et al., 2010; Bruno et al., 2014; McCleary et al., 2014; Ruane et al., 2018), indicating that marine fish may be a reservoir of marine SAV. It has been also detected in sea lice (*Lepeophtheirus salmonis*) (Petterson et al., 2009).

Although SAV infection and the clinical condition have been experimentally induced in the freshwater stages of Atlantic salmon (Boucher et al., 1995; McLoughlin et al., 1995; Desvignes et al., 2002; Cano et al., 2015), there are no reports of clinical field cases. Therefore, the aim of this study is to report the first outbreak of a SAV subtype I infection with associated clinical presentation in the freshwater culture of Atlantic salmon.

2. Material and methods

2.1. Site information

The hatchery facility is located on the west coast of Scotland less than one mile from a sea water loch that contains several A. salmon aquaculture facilities. The fish were held in a recirculation system consisting of four tanks, however, only three tanks were populated with fish. The water is supplied from a nearby river and treated with ozone and ultraviolet (UV) disinfection at the inlet point and on-site the water is filtered by both drum and biofilters. The site was populated with approximately 1,400,000 S0 Atlantic salmon with a mean weight ranging from 10 to 16 g (Fig 1). These fish were supplied by another hatchery as fry in May 2018.

An increased mortality was reported to the Marine Scotland Science, Fish Health Inspectorate, on 29 May 2018. An inspector visited the site on 31 May 2018. Mortalities were reported as 2.16% in week 21 and 11.67% in week 22 (the week of the visit) and then decreased to 0.55% in week 23. The site suspected an IPN outbreak and raised the water temperature to 21 °C for 48 h to combat the suspected IPN outbreak. The inspector observed large numbers of moribund, dead and dying fish in the recirculation systems.

2.2. Sampling, histology examination and bacteriology

A total of five moribund and lethargic fish were randomly removed from the outflow of the three populated tanks for diagnostic sampling. Fish were killed by percussive stunning prior to sampling. Skin, gill, heart, liver, kidney, spleen, gut and pancreas were sampled and placed in 10% neutral buffer formalin, processed by standard paraffin wax technique, cut (3 μm) using rotary microtome and stained with routine haematoxylin and eosin (H & E) protocols. Sections were examined under light microscopy (Olympus BX 45) and images captured using CellˆD2640 software (Cell Software). Kidney and spleen were also inoculated onto appropriate media, tryptone soya agar (TSA; Oxoid CM0131) and FLP agar (Cepeda et al., 2004) for the isolation of bacteria but no significant bacterial growth was observed.

2.3. Virological examination

2.3.1. Molecular virological testing

One pool of heart and kidney tissue, totaling approximately 0.5 g, from the five fish sampled was collected into RNAlater (Ambion, Inc.) for molecular analysis and transported at 4 °C to the Marine Laboratory, Aberdeen. RNA extraction and quantitative reverse transcription PCR (RT-qPCR) were performed as described by Snow et al., (2010). The SAV Q_NSP1 (Hodneland and Endresen, 2006) assay was used alongside salmonid ELF1α GIM-2 (Sepúlveda et al., 2013) endogenous control assay. A fragment of the E2 gene was then amplified and sequenced from the extracted RNA material using reverse transcription-PCR. The product was purified (ExoSAp-IT PCR product cleanup ThermoFisher) and sent to DNA Sequencing and Services (MRC I PPU, School of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) to be sequenced. The sequence has been submitted to Genbank and assigned accession number MH70676. Mega 6.06 was used to conduct the phylogenetic analysis (Tamura et al., 2013) using multiple sequence alignments (357 nucleotides) with SAV reference sequences assembled using CLUSTAL W (Chenna et al., 2003). The best model for maximum likelihood analysis was determined as General Reversible Mitochondrial +Freq model (Adachi and Hasegawa, 1996) with gamma distribution by Bayesian Information Criterion scores. The tree was bootstrapped using 1000 replicates. The extracted RNA was also screened for infectious pancreatic necrosis virus (IPNV) according to Taksdal et al. (2001).

2.3.2. Virology cell culture isolation

Approximately 1 g of heart, kidney and spleen tissue from the aforementioned 5 fish were sampled and placed into one pool and transported to the Marine Laboratory in 9 ml virus transport media (Liebovitz L-15, Sigma; 10% new born calf serum, Invitrogen; gentamicin at 50 mg l^{-1}, Invitrogen; poly myxin B at 10000 U ml^{-1}, Sigma)

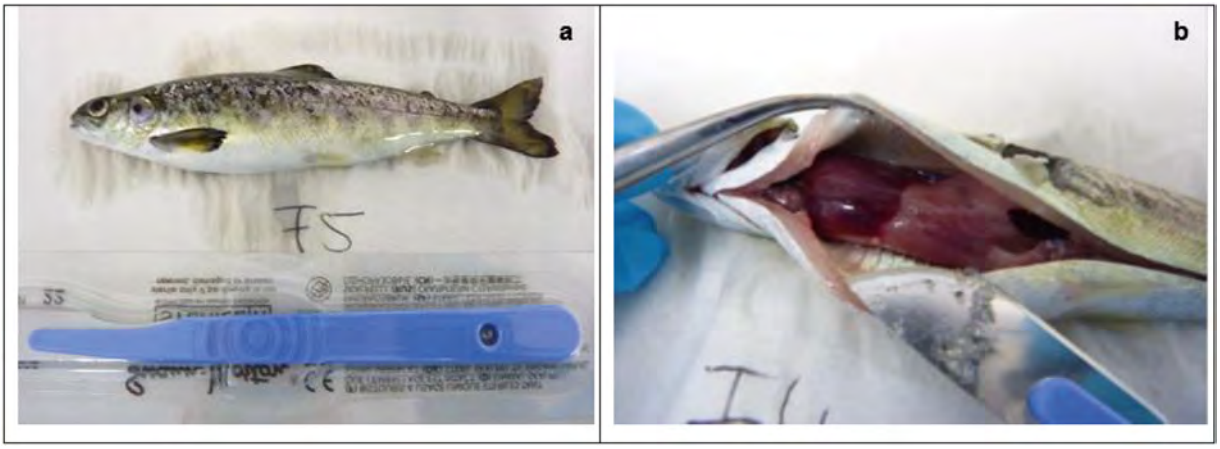


Fig. 1. a) Freshwater Atlantic salmon parr; b) Petechial haemorrhaging on the liver and the pyloric caeca.

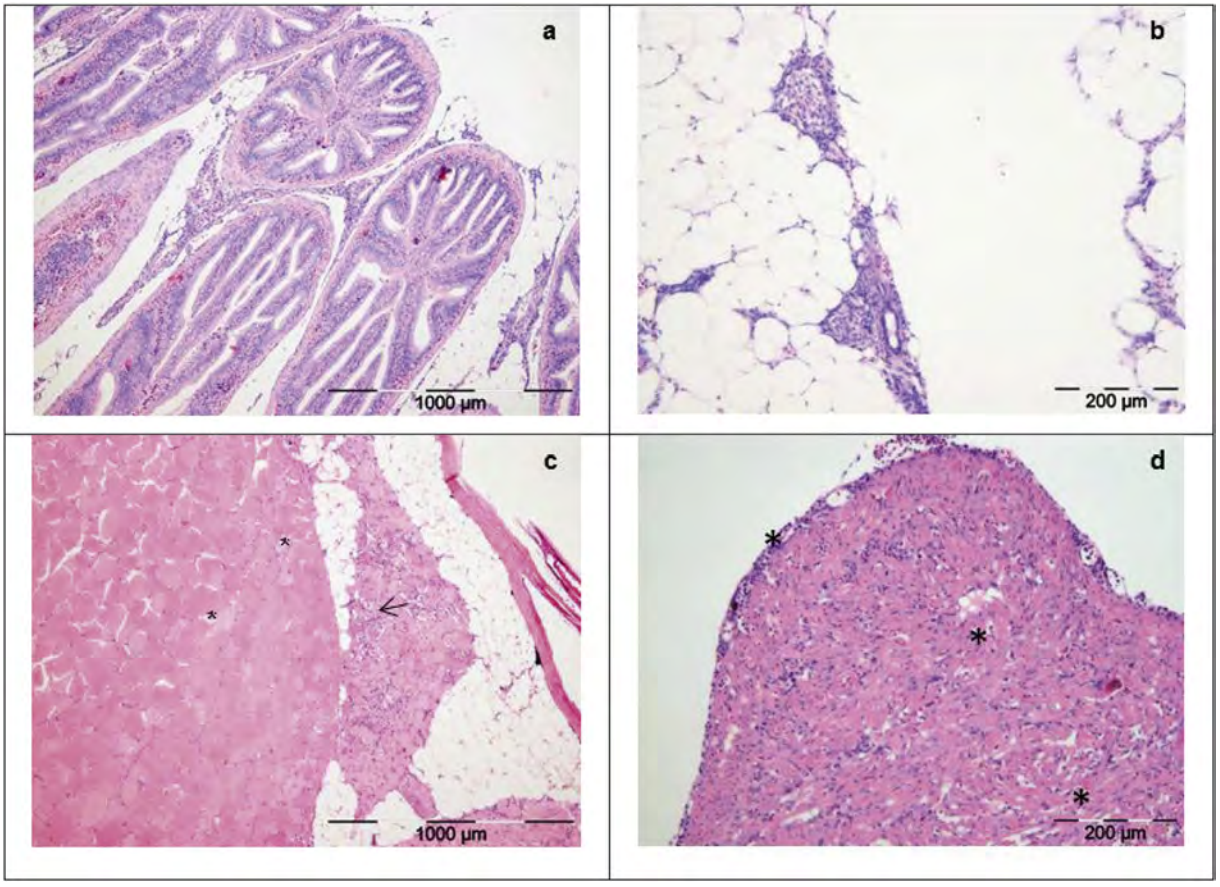


Fig. 2. Histopathology of Atlantic salmon parr showing clinical signs. (a,b) Cross-section showing absence of pancreatic acinar cells. (c) Cross-section showing red and white skeletal muscle lesions; degeneration of myocyte – white muscle (*), red skeletal muscle necrosis (arrow). (d) Cross-section demonstrating multifocal cardiomyocytic necrosis with cells presenting shrunken eosinophilic cytoplams and pyknotic nuclei and epicarditis (*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 4 °C. Twelve well plates containing monolayers of CHSE-214 cells were inoculated according to Fryer et al. (1965). The cell monolayers were at 60 to 80% confluence and 24 to 48 h old. The inoculated cells were then incubated at 15 °C and checked every seven days for the appearance of viral cytopathic effect (CPE). On day 14 and 28, the cultures were sub-cultivated and on day 41, the cells were scraped into RTL Plus buffer (Qiagen) containing 0.1% β-mercaptoethanol (Sigma) for SAV RT-qPCR confirmation.

3. Results

3.1. Case description and macroscopic observations

The mean weight of the Atlantic salmon ranged from 10 to 16 g. Externally, one fish showed some haemorrhaging to the base of the pectoral fin and had a flared opercula. The gills appeared zoned in colour in all five fish. Internally, bloody ascites was observed in two fish. Petechial haemorrhaging on the liver and the pyloric caeca was observed in two fish and one of them also showed darker than normal

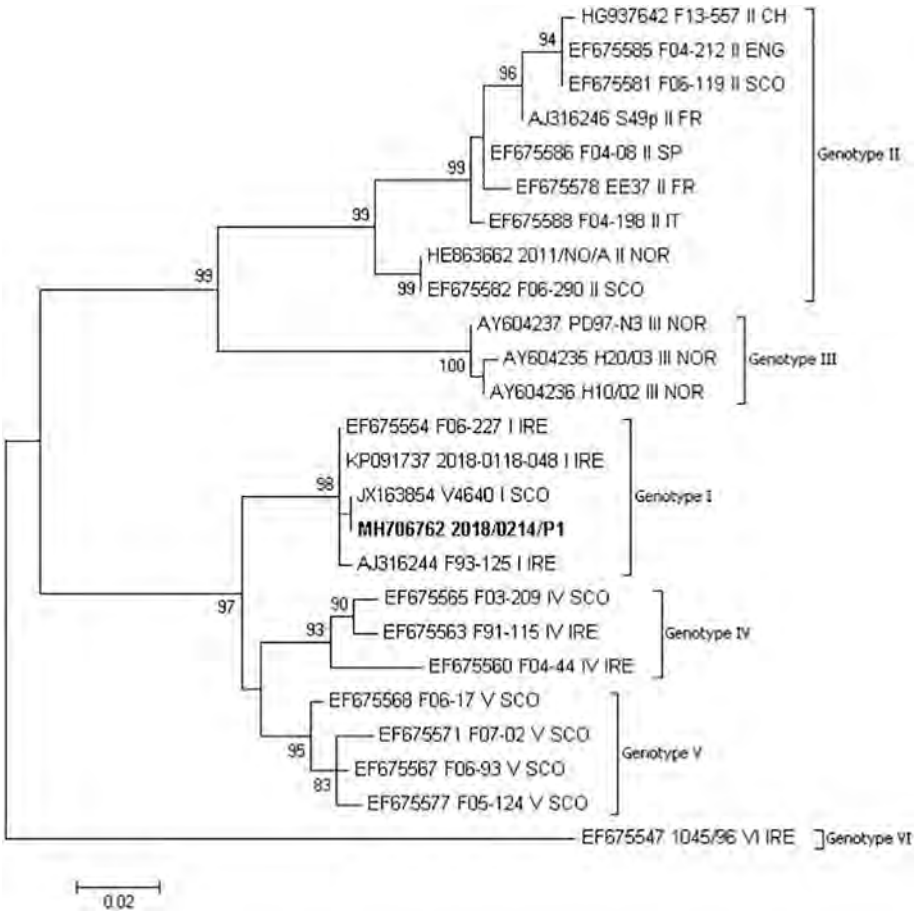


Fig. 3. Phylogenetic analysis of partial E2 gene sequences (357 nts). The percentage of trees in which the associated taxa are clustered together is shown next to the branches. Values < 75% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Sequences are named by accession number, isolate name, genotype and geographic origin - CH (Switzerland), SCO (Scotland), ENG (England), FR (France), SP (Spain), IT (Italy), NOR (Norway) or IRE (Republic of Ireland). The Salmonid alphavirus subtype I isolated from clinically-diseased Atlantic salmon, *Salmo salar*, in freshwater is highlighted in bold text.

liver colour. Yellow pseudo faeces were present in two fish, with no food present in the guts of three fish. The kidney appeared slightly grey in two Atlantic salmon.

3.2. Histopathology examination

Histopathological examination revealed marked loss of pancreatic acinar cells (Fig. 2a, b) and three fish only showed scattered, small pockets of acinar cells. The skeletal muscle showed mild to moderate, multifocal to diffuse degeneration of red skeletal muscle (Fig. 2c). White skeletal muscle also demonstrated features of myofiber degeneration but to a lesser extent (Fig. 2c). Multifocal cardiomyocyte necrosis, showing cells with shrunken, eosinophilic cytoplasm and pyknotic nuclei (Fig. 2d) and multifocal epicarditis were also noted in all fish. Other histopathological changes were mild to moderate hepatocyte vacuolation.

3.3. Virological examination

The pool of heart and kidney tissue taken from the five fish tested IPNV negative by RT-qPCR, however, the same pool screened positive (Cp ~21.5) for SAV by RT-qPCR and was subsequently confirmed as SAV by partial E2 gene sequencing. The virus was classified as SAV subtype I by phylogenetic analysis (Fig. 3) and grouped with other strains from Scotland and Ireland. Phylogenetically this SAV strain was more closely related to the Scottish isolate, JX163854 V4640 I SCO, that was originally isolated from marine reared Atlantic salmon. Salmonid alphavirus was also isolated from CHSE-214 cells after two passages where a mild cytopathic effect was noted and then confirmed as SAV by RT-qPCR.

4. Discussion

It is known that the freshwater stages of Atlantic salmon e.g. fry, parr and smolts, are experimentally susceptible to SAV infection and that the clinical PD condition, with associated typical histopathological lesions in the heart, pancreas and skeletal muscle have been observed from experimental studies (Boucher et al., 1995; McLoughlin et al., 1995; Desvignes et al., 2002; Cano et al., 2015). SAV subtype I was also previously detected from tissue samples collected from rainbow trout at several Scottish freshwater sites by Lester et al., (2011) as part of a research based surveillance survey performed between 2006 and 2007. However, no observations of clinical PD were reported from this study. This survey also included Atlantic salmon freshwater sites that were negative for SAV. To the authors knowledge and in accordance with the PD review paper published by Jansen et al., 2017, this appears to be the first published description of a clinical field case of SAV with pathology consistent with PD in freshwater Atlantic salmon. This case showed typical histopathological lesions of clinical PD in the pancreas, heart and skeletal muscle (McLoughlin and Graham, 2007). The phylogenetic analysis of the virus places it in the subtype of SAV I, closely related to isolates previously reported in Scotland and Ireland. This is not a surprising finding, as SAV subtype I is commonly found in Scottish clinical PD cases in the marine environment (Matejusova et al., 2013; Jansen et al., 2017).

The origin of the SAV introduction at this hatchery is unknown. The site staff suspected an IPN outbreak, a condition frequently diagnosed at salmonid freshwater sites, from histology results taken prior to the FHI visit. The mortalities were elevated and water temperature raised to 21 °C for 48 h in an attempt to control the impact of the disease. The RT-qPCR screening results from the pooled tissues were negative for IPNV and positive for SAV which was then confirmed to be SAV I by

partial E2 gene sequence analysis. As the pooled sample consisted of tissue material from five fish, it is impossible to determine the number of individuals that were SAV positive in the pool. However, histopathology examination showed alterations in pancreas, heart and skeletal muscle consistent with PD in all five fish. The red skeletal muscle was more affected than the white skeletal muscle which was also described by Cano et al. (2015) in experimentally induced PD caused by SAV I. The isolation of SAV from cell culture demonstrated that the SAV virions were active.

According to Jansen et al., 2017, the risk of SAV being transmitted vertically is considered insignificant. Conversely, the risk of SAV being transmitted horizontally is well studied (Kristoffersen et al., 2009; Jansen et al., 2010). These authors suggested that the infection pressure of SAV in endemic regions is a risk factor for the virus spread and that horizontal transmission of the virus happens most likely after seawater transfer. Kristoffersen et al. (2009) also suggested that bio-security measures are important to reduce the horizontal transmission of SAV and consequently the occurrence of PD outbreaks. There are no reports indicating that the stocked fish originated from SAV infected broodfish and as mentioned above the risk of vertical transmission is considered minimal. However, it is not a legal obligation to screen broodfish for SAV in Scotland.

The freshwater hatchery is located less than a mile from a seawater loch where several Atlantic salmon seawater sites are located and PD has been historically diagnosed in this management area. In addition, SAV I was previously confirmed on marine A. salmon production sites in this geographical area. The potential for horizontal transmission of SAV from seawater production to the freshwater site via movement of staff and equipment exists. However, the FHI did not obtain evidence that this route of transmission occurred. It would also be interesting to gather information on the health status and production indicators (e.g. growth rate and feed conversation ratio) of this stock of fish after they were transferred to seawater. In addition, whole genome sequencing analysis of this SAV I strain would be interesting to better understand how genetically related this virus is to the SAV I previously detected from freshwater rainbow trout (Lester et al., 2011) or in marine salmonids, including strains that were detected in the seawater management area adjacent to this freshwater site (Matejusova et al., 2013; Bruno et al., 2014). Until now, PD outbreaks in freshwater Atlantic salmon have only been described from experimental challenge trials. This is the first report of a field case of a PD outbreak in Atlantic salmon in freshwater and as such, is a very significant development in the epidemiology of PD.

Acknowledgements

We acknowledge the Scottish Government for funding this work (contract number AQ03Q0) and we also would like to thank MSS diagnostic staff for the co-operation during the diagnostic investigation.

References

Adachi, J., Hasegawa, M., 1996. Model of amino acid substitution in proteins encoded by mitochondrial DNA. J. Mol. Evol. 42, 459–468. https://doi.org/10.1007/BF02498640.

Aunsmo, A., Valle, P.S., Sandberg, M., Midtlying, P.J., Bruheim, T., 2010. Stochastic modeling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (*Salmo salar* L.). Prev. Vet. Med. 93, 233–241. https://doi.org/10.1016/j.prevetmed.2009.10.001.

Boucher, P., Baudin Laurencin, F., 1994. Sleeping disease (SD) of salmonids. Bull. Eur. Assoc. Fish Pathol. 14 (5), 179–180.

Boucher, P., Raynard, R.S., Houghton, G., Laurencin, F.B., 1995. Comparative experimental transmission of pancreas disease in Atlantic salmon, rainbow trout and brown trout. Dis. Aquat. Org. 22 (1), 19–24. https://doi.org/10.1111/j.1365-2761.1996.tb00708.x.

Bratland, A., Nylund, A., 2009. Studies on the possibility of vertical transmission of Norwegian salmonid Alphavirus in production of Atlantic salmon in Norway. J. Aquat. Anim. Health 21, 173–178. https://doi.org/10.1577/H08-038.1.

Bruno, D.W., Noguera, P.A., Black, J., Murray, W., Macqueen, D.J., Matejusova, I., 2014. Identification of a wild reservoir of salmonid alphavirus in common dab, *Limanda limanda*, with emphasis on virus culture and sequencing. Aquac. Environ. Interact. 5, 89–98. https://doi.org/10.3354/aei00097.

Cano, I., Joiner, C., Bayley, A., Rimmer, G., Bateman, K., Feist, S.W., Stone, D., Paley, R., 2015. An experimental means of transmitting pancreas diseases in Atlantic salmon *Salmon salar* L. fry in freshwater. J. Fish Dis. 38, 271–281. https://doi.org/10.1111/jfd.12310.

Cepeda, C., Garcia-Marquez, S., Santos, Y., 2004. Improved growth of *Flavobacterium psychrophilum* using a new culture medium. Aquaculture 238, 75–82. https://doi.org/10.1016/j.aquaculture.2004.05.013.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., Thompson, J.D., 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31, 3497–3500.

Crockford, T., Menzies, F.D., McLoughlin, M.F., Wheatley, S.B., Goodall, E.A., 1999. Aspects of epizootiology of pancreas disease in farmed Atlantic salmon *Salmo salar* in Ireland. Dis. Aquat. Org. 36, 113–119. https://doi.org/10.3354/dao036113.

Desvignes, L., Quentel, C., Lamour, F., Le Ven, A., 2002. Pathogenesis and immune response in Atlantic salmon (*Salmo salar* L.) parr experimentally infected with salmon pancreas disease virus (SPDV). Fish Shellfish Immunol. 12, 77–95. https://doi.org/10.1006/fsim.2001.0356.

Fringuelli, E., Rowley, H.M., Wilson, J.C., Hunter, R., Rodger, H., Graham, D.A., 2008. Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. J. Fish Dis. 31, 811–824. https://doi.org/10.1111/j.1365-2761.2008.00944.x.

Fryer, J.L., Yusha, A., Pilcher, K.S., 1965. The in vitro cultivation of tissue and cells of Pacific salmon and steelhead trout. Ann. N. Y. Acad. Sci. 126, 566–586. https://doi.org/10.1111/j.1749-6632.1965.tb14303.x.

Graham, D.A., Fringuelli, E., Wilson, C., Rowley, H.M., Brown, A., Rodger, H., McLouglin, M.F., McManus, C., Casey, E., McCarthy, L.J., Ruane, N.M., 2010. Prospective longitudinal studies of salmonid alphavirus infections on two Atlantic salmon farms in Ireland; evidence for viral persistence. J. Fish Dis. 33, 123–135. https://doi.org/10.1111/j.1365-2761.2009.01096.x.

Graham, D.A., Fringuelli, E., Rowley, H.M., Cockerill, D., Cox, D.I., Turnbull, T., Rodeger, H., Morris, D., Loughlin, M.F.Mc, 2012. Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon, Salmo salar L., in Scotland and Ireland. J. Fish Dis. 35, 755–765. https://doi.org/10.1111/j.1365-2761.2012.01401.x.

Hodneland, K., Endresen, C., 2006. Sensitive and specific detection of Salmonid alphavirus using real-time PCR (TaqMan®). J. Virol. Methods 131, 184–192. https://doi.org/10.1016/j.jviromet.2005.08.012.

Jansen, M.D., Taksdal, T., Wasmuth, M.A., Gjerset, B., Brun, E., Olsen, A.B., Breck, O., Sandberg, M., 2010. Salmonid alphavirus (SAV) and pancreas disease (PD) in Atlantic salmon, *Salmo salar* L., in freshwater and seawater sites in Norway from 2006 to 2008. J. Fish Dis. 33, 391–402. https://doi.org/10.1111/j.1365-2761.2009.01131.x.

Jansen, M.D., Jensen Bang, B., McLoughlin, M.F., Rodger, H.D., Taksdal, T., Sindre, H., Graham, D.A., Lillehaug, A., 2017. The epidemiology of pancreas disease in salmonid aquaculture: a summary of the current state of knowledge. J. Fish Dis. 40, 141–155. https://doi.org/10.1111/jfd.12478.

Kilburn, R., Murray, A.G., Hall, M., Bruno, D.W., Cockerill, D., Raynard, R.S., 2012. Analysis of a company's production data to describe the epidemiology and persistence of pancreas disease in Atlantic salmon (*Salmo salar* L.) farms off Western Scotland. Aquaculture 368-369, 89–94. https://doi.org/10.1016/j.aquaculture.2012.09.004.

Kongtorp, R.T., Stene, A., Andreassen, P.A., Aspehaug, V., Graham, D.A., Lyngstad, T.M., Olsen, A.B., Olsen, R.S., Sandberg, M., Santi, N., Wallace, C., Breck, O., 2010. Lack of evidence for vertical transmission of SAV 3 using gametes of Atlantic salmon, *Salmo salar* L., exposed by natural and experimental routes. J. Fish Dis. 33, 879–888. https://doi.org/10.1111/j.1365-2761.2010.01190.x.

Kristoffersen, A.B., Viljugrein, H., Kongtorp, R.T., Brun, E., Jansen, P.A., 2009. Risk factors for pancreas disease (PD) outbreaks in farmed Atlantic salmon and rainbow trout in Norway during 2003–2007. Prev. Vet. Med. 90, 127–136. https://doi.org/10.1016/j.prevetmed.2009.04.003.

Larsson, T., Krasnov, A., Lerfall, J., Taksdal, T., Pedersen, M., Mørkøre, T., 2012. Fillet quality and gene trasncriptome profiling of heart tissue of Atlantic salmon with pancreas disease (PD). Aquaculture 330-333, 82–91. https://doi.org/10.1016/j.aquaculture.2011.12.016.

Lerfall, J., Larsson, T., Birkeland, S., Taksdal, T., Dalgaard, P., Afanasyev, S., Bjerke, M.T., Mørkøre, T., 2012. Effects of pancreas disease (PD) on quality attributes of raw and smoked fillets of Atlantic salmon (*Salmo salar* L.). Aquaculture 324-325, 209–217. https://doi.org/10.1016/j.aquaculture.2011.11.003.

Lester, K., Black, J., Bruno, B.D., 2011. Prevalence of salmonid alphavirus in Scottish fish farms from 2006 to 2007. Bull. Eur. Assoc. Fish Pathol. 31, 199–204.

Matejusova, I., Lester, K., Li, Z., Bravo, J., Bland, F., Collet, B., 2013. Comparison of complete polyprotein sequences of two isolates of salmon alphavirus (SAV) type I and their behaviour in a salmonid cell line. Arch. Virol. 158, 2143–2146. https://doi.org/10.1007/s00705-013-1689-4.

McCleary, S., Giltrap, M., Henshilwood, K., Ruane, N.M., 2014. Detection of salmonid alphavirus RNA in Celtic and Irish Sea flatfish. Dis. Aquat. Org. 109, 1–7. https://doi.org/10.3354/dao02719.

McLoughlin, M.F., Graham, D.A., 2007. Alphavirus infections in salmonids – a review. J. Fish Dis. 30, 511–531. https://doi.org/10.1111/j.1365-2761.2007.00848.x.

McLoughlin, M.F., Nelson, R.T., McCormick, J.I., Rowley, H., 1995. Pathology of experimental pancreas disease in freshwater Atlantic salmon parr. J. Aquat. Anim. Health 7 (2), 104–110. https://doi.org/10.1577/1548-8667(1995)007<0104:POEPDI>2.3.CO;2.

McLoughlin, M.F., Nelson, R.T., McCormick, J.I., Rowley, H.M., Bryson, D.G., 2002. Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, *Salmo salar* L. J. Fish Dis. 25, 33–43. https://doi.org/10.

1046/j.1365-2761.2002.00334.x.
Munro, A.L.S., Ellis, A.E., McVicar, A.H., McLay, H.A., Needham, E.A., 1984. An exocrine pancreas disease of farmed Atlantic salmon in Scotland. Helgoländer Meeresun. 37 (1/4), 571–586.
Nelson, R.T., McLoughlin, M.F., Rowley, H.M., Platten, M.A., McCormick, J.I., 1995. Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. Dis. Aquat. Org. 22 (1), 25–32. https://doi.org/10.3354/dao022025.
OIE, 2018. Infection with salmonid alphavirus. Diseases of fish (section 2.3). In: Manual of Diagnostic Tests for Aquatic Animals, . http://www.oie.int/index.php?id=2439&L=0&htmfile=chapitre_salmonid_alphavirus.htm.
Petterson, E., Sandberg, M., Santi, N., 2009. Salmonid alphavirus associated with *Lepeophtheirus salmonis* (Copepoda: Caligidae) from Atlantic salmon, *Salmo salar* L. J. Fish Dis. 32, 477–479. https://doi.org/10.1111/j.1365-2761.2009.01038.x.
Rodger, H., Mitchell, S., 2007. Epidemiological observations of pancreas disease of farmed Atlantic salmon, *Salmo salar* L., in Ireland. J. Fish Dis. 30, 157–167. https://doi.org/10.1111/j.1365-2761.2007.00799.x.
Ruane, N., Graham, D., Rodger, H., 2008. Pancreas Disease in Farmed Salmon – Health Management and Investigations at Irish Sites 2005–2008. Marine Environment and Health Series, No. 34. Marine Institute, Galway, Ireland.
Ruane, N., Swords, D., Morrissey, T., Geary, M., Hickey, C., Collins, E.M., Geoghegan, F., Swords, F., 2018. Isolation of salmonid alphavirus subtype 6 from wild-caught ballan wrasse, *Labrus bergylta* (Ascanius). J. Fish Dis. 41, 1643–1651. https://doi.org/10.1111/jfd.12870.
Sepúlveda, D., Bohle, H., Labra, Á., Grothusen, H., Marshall, S.H., 2013. Design and evaluation of a unique RT-qPCR assay for diagnostic quality control assessment that is applicable to pathogen detection in three species of salmonid fish. BMC Vet. Res. 9, 183.
Snow, M., Black, J., Matejusova, I., McIntosh, R., Barreto, E., Wallace, I.S., Bruno, D.W., 2010. Evidence for the detection of salmonid alphavirus (SAV) RNA in wild marine fish caught in areas remote from aquaculture activity: implications for the origins of salmon pancreas disease (SPD) in aquaculture. Dis. Aquat. Org. 91, 177–188. https://doi.org/10.3354/dao02265.
Steinbauer, P., Stagg, H.E.B., Böhm, B., Schade, B., Kappe, E., McIntosh, R., Munro, E.S., 2019. First reported outbreak of sleeping disease in Alsatian charr (*Salvelinus fontinalis* x *Salvelinus alpinus* hybrid). Bull. Eur. Assoc. Fish Pathol. 39, 3–13.
Taksdal, T., Dannevig, B.H., Rimstad, E., 2001. Detection of infectious pancreatic necrosis (IPN)-virus in experimentally infected Atlantic salmon parr by RT-PCR and cell culture isolation. Bull. Eur. Assoc. Fish Pathol. 21, 214–219.
Taksdal, T., Olsen, A.B., Bjerkas, I., Hjortas, M.J., Dannevig, B.H., Graham, D.A., McLoughlin, M.F., 2007. Pancreas disease in farmed Atlantic samon, *Salmo salar* L., and rainbow trout, *Oncorhyncus mykiss* (Walbaum), in Norway. J. Fish Dis. 30, 545–558. https://doi.org/10.1111/j.1365-2761.2007.00845.x.
Taksdal, T., Wiik-Nielsen, J., Birkeland, S., Dalgaard, P., Mørkøre, T., 2012. Quality raw and smoked fillets from clinicaly healthy Atlantic salmon, *Salmo salar* L., following an outbreak of pancreas disease (PD). J. Fish Dis. 35, 897–906. https://doi.org/10.1111/j.1365-2761.2012.01428.x.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.

Vol. 42: 53–59, 2000 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published August 10

Ultraviolet irradiation inactivates the waterborne infective stages of *Myxobolus cerebralis*: a treatment for hatchery water supplies

R. P. Hedrick[1,*], T. S. McDowell[1], G. D. Marty[2], K. Mukkatira[1], D. B. Antonio[1], K. B. Andree[1], Z. Bukhari[3], T. Clancy[3]

[1]Department of Medicine and Epidemiology and [2]Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, California 95616, USA

[3]Clancy Environmental Consultants, Inc., St. Albans, Vermont 05478, USA

ABSTRACT: The effects of ultraviolet (UV) irradiation on the viability of the waterborne triactinomyxon stages of *Myxobolus cerebralis* were evaluated by vital staining and the infectivity for juvenile rainbow trout *Oncorhynchus mykiss*. A dose of 1300 mW s cm^{-2} was required to inactivate 100% of the triactinomyxons held under a static collimated beam of UV as determined by vital staining. Juvenile rainbow trout were protected from infections with *M. cerebralis* when exposed to 14 000 or 1400 triactinomyxon spores per fish that had been treated with the collimating beam apparatus (1300 mW s cm^{-2}). Among all fish receiving UV-treated triactinomyxons, none had clinical signs of whirling disease, or evidence of microscopic lesions or spores of *M. cerebralis* after 5 mo at water temperatures of 15°C. In contrast, 100% of the fish receiving the higher dose of untreated triactinomyxons developed clinical signs of whirling disease and both microscopic signs of infection and spores were detected in all of the high and low dose trout receiving untreated triactinomyxon exposures. Two additional trials evaluated the *Cryptosporidium* Inactivation Device (CID) for its ability to treat flow-through 15°C well water to which triactinomyxons were added over a 2 wk period. CID treatments of a cumulative dose exceeding 64 000 triactinomyxons per fish protected juvenile rainbow from infections with *M. cerebralis*. Rainbow trout controls receiving the same number of untreated triactinomyxons developed both microscopic lesions and cranial spore concentrations up to $10^{4.6}$ per ½ head, although no signs of clinical whirling disease were observed. UV (126 mW s cm^{-2}, collimated beam apparatus) was also effective in killing *Flavobacterium psychrophilum*, the agent causing salmonid bacterial coldwater disease, as demonstrated by the inability of bacterial cells to grow on artificial media following UV treatment.

KEY WORDS: Whirling disease · *Flavobacterium psychrophilum* · Ultraviolet · Disease control

INTRODUCTION

Whirling disease among salmonid fish is caused by the myxozoan parasite *Myxobolus cerebralis* (Hofer 1908). The disease, once thought to be problematic only among hatchery-reared salmonids (Halliday 1976, Hoffman 1990), has now been identified as a major cause of declines among certain wild trout populations in the intermountain west of the USA (Nehring & Walker 1996, Vincent 1996). The disease has been effectively managed in hatchery-reared salmonids by preventing or reducing the exposure of young susceptible fish to the infectious stages or triactinomyxons as released from infected oligochaetes (Hoffman 1990). This has been accomplished by rearing young fish in well water to avoid exposures to the parasite until fish are older and more resistant, or by modifying pond designs to minimize habitat for the oligochaete host (Schaperclaus 1986, Wolf 1986).

*E-mail: rphedrick@ucdavis.edu

While clinical disease manifestations may be controlled by these measures, fish are infected and the parasite develops to form spores in the skeletal elements. The stocking of subclinically infected fish is one principal method responsible for the spread of whirling disease (Hoffman 1970, Modin 1998).

The studies of Wolf & Markiw (1984) demonstrated the 2-host life cycle for the parasite and identified the previously unknown waterborne infectious stages of *Myxobolus cerebralis*: the triactinomyxons. The spore stages produced in the fish are extremely resistant to physical and chemical inactivation (El-Matbouli et al. 1992). In contrast, the triactinomyxon stages released into the water from infected oligochaetes are shorter lived and more susceptible to inactivation (Markiw 1992a). Removing or inactivating these waterborne infectious stages is viewed as an essential approach to controlling infections with *M. cerebralis* among salmonids when surface waters must be utilized for hatchery rearing. Hoffman (1974, 1975) demonstrated that ultraviolet light (UV) treatments were effective in protecting rainbow trout from whirling disease even though the waterborne infectious stages were at that time unknown. Hoffman (1974, 1975) found UV treatments at doses ranging from 18 to 112 mW s cm^{-2} provided partial to complete protection from *M. cerebralis* infections. These initial studies demonstrated the potential of UV treatments but did not establish minimum lethal doses required to inactivate the triactinomyxon form of *M. cerebralis.* In more recent years, sophisticated and much more powerful systems for treatment of water with UV (for municipal water supplies) have been developed (Campbell et al. 1995, Clancy et al. 1998, Bukhari et al. 1999). The *Cryptosporidium* Inactivation Device (CID) technology is one example of these newer approaches to the use of disinfection of water using UV light (Clancy et al. 1998). This device uses a combination of UV and nominal 2 µm sintered stainless steel screens for effective trapping and inactivation of the human pathogen *Cryptosporidium* in municipal water supplies. In this study we describe the use of a collimating beam apparatus to determine the dose response curves for the triactinomyxon stages of *M. cerebralis* as evaluated by vital staining and infectivity for rainbow trout *Oncorhynchus mykiss*. In addition, we examined the dose response of *Flavobacterium psychrophilum*, the agent causing salmonid bacterial coldwater disease, to UV irradiation (Bernardet et al. 1996). We then tested the efficacy of UV treatments as provided by the CID to inactivate triactinomyxons introduced into a flow-through system to aquaria containing rainbow trout. The results of these studies and the application of these approaches to eliminate *M. cerebralis* from hatchery water supplies are described here.

METHODS

Laboratory propagation of parasite stages. The triactinomyxons of *Myxobolus cerebralis* were propagated in the laboratory from known susceptible oligochaete populations infected with spores extracted from rainbow trout *Oncorhynchus mykiss* tissues by the plankton centrifuge procedure as described by Andree et al. (1998). Triactinomyxons from infected oligochaete populations were harvested every 2 to 3 d as required for evaluating the efficacy of UV light treatments in destroying the infectivity of the triactinomyxons for fish. Two UV inactivation procedures were employed, the first utilized a collimating beam apparatus for establishing dose response curves by vital staining and destruction of infectivity for rainbow trout (static UV treatments). The second procedure involved continuous treatments of large water volumes with the CID, a unit designed to treat municipal water supplies (Clancy et al. 1998). During flow-through trials with the CID, triactinomyxons were periodically added to the water supply before entering the CID; treated water was dispensed to tanks containing young rainbow trout.

UV dose response curves—collimating beam. A collimating beam apparatus (kindly supplied by Safe Water Solutions L.L.C., Scottsdale, Arizona) with 4 identical beams of UV light from 1 light tube was used to direct UV light onto 4 petri dishes mounted on insulated magnetic stir plates (Bukhari et al. 1999). The intensity of the UV irradiation on bisecting *x*- and *y*-axes of the dishes was measured with a radiometer and sensor (Bukhari et al. 1999). The measured values were used to calculate an average irradiance over the surface of the dish.

The effects of UV exposure on triactinomyxons suspended in 20 ml of water in each petri dish under 3 of the replicate UV beams was compared with 2 control dishes shielded from the radiation. The solutions were mixed gently throughout the treatment with magnetic stir bars. In the initial dose response experiments 0.1 ml aliquots were removed from each dish at selected time points for later analysis by vital staining. The appropriate dosage for complete inactivation of the triactinomyxons was then determined. Subsequently, larger numbers of parasites were irradiated and tested for their ability to infect young rainbow trout.

Vital staining of triactinomyxons. An adaptation of the vital stain originally used for triactinomyxons of *Myxobolus cerebralis* by Markiw (1992b) and as described by Wagner (1998) was used to determine potential viability of the parasite after exposure to UV irradiation. A 100 µl aliquot from the parasite suspension was mixed on a microscope slide with 50 µl of a

52 mg l^{-1} propidium iodide solution and 50 µl of fluorescein diacetate (100 µl of 5 mg ml^{-1} stock solution added to 8.3 ml water). A coverslip was added and the slide was held at least 45 min at 4°C before examination with a microscope equipped with the proper excitation and barrier filters. Under these conditions viable sporoplasm cells in the triactinomyxons stained green while those that were inactivated stained red.

Effects of static UV treatments on infectivity for trout. Triactinomyxons for the fish infectivity study were aliquoted to 8 petri dishes: 4 dishes with 1.4×10^6 and 4 with 1.40×10^5 triactinomyxons provided a low (1400 per fish) and high (14 000 per fish) dose exposure of rainbow trout. Two dishes from each group were treated with UV for the equivalent of 1300 mWs cm^{-2} at 20°C. The irradiance under the beam was 135 µW cm^{-2}; therefore, approximately 3 h was required to achieve the needed dose. Two dishes from each group were held under the same conditions but did not receive UV treatment (Low and High). Groups of 40 rainbow trout (0.2 g) were then exposed to the contents of each petri dish representing the 4 treatment groups in 15°C well water. An additional 2 groups of 40 fish received no exposures to triactinomxyons (unexposed control). The fish were then transferred to 20 l aquaria receiving 15°C well water at a rate of 3 l min^{-1}. Details on the exposure protocols and fish maintenance are described by Hedrick et al. (1999a). After 5 wk, 5 fish from each unexposed group and each group receiving high dose triactinomyxon exposures with or without UV treatments were removed and examined by PCR for the presence of *Myxobolus cerebralis* infection (Andree et al. 1998). At 5 mo post-exposure the experiment was terminated. Ten fish from each tank were examined individually for spore concentrations in cranial cartilage and microscopic lesions in stained tissue sections associated with *M. cerebralis* (Thoesen 1994) as described by Hedrick et al. (1999b). Remaining fish in all groups exposed to UV-treated parasites were analyzed as 5 fish pools for the presence or absence of the spores in cranial cartilage.

Effects of CID UV treatments on infectivity for trout. The CID unit was installed directly into the Fish Disease Laboratory at the University of California Davis such that all water delivered to six 130 l tanks received treatment prior to contacting the fish. The CID unit irradiates water that flows through its 2 µm filters, and particulates trapped on the filter are treated with high-energy UV irradiation. The filtration and irradiation cycles between 2 chambers, and filters are back flushed at the end of each cycle to maintain flow rates. During triactinomyxon trials, the flow of 15°C well water through the machine was 18 l min^{-1}, with 3 l min^{-1} flowing into each of the 6 test aquaria. At all other times the flow through the machine was 131 l min^{-1}, with flow in excess of the 18 l min^{-1} bypassing the fish tanks into a recirculating loop with a 650 l circular holding tank.

To introduce parasites into the treatment system, tractinomyxons were suspended in 4 l of water and metered into the intake pipe of the CID with a pump over a period of approximately 12 min. This time frame overlapped the cycle between the 2 chambers of the filtration-irradiation units to ensure that parasites were flowing through the complete range of the machine's function. The UV dosage on the CID was set at 4000 mWs cm^{-2} per chamber, a significant excess over the 1300 mWs cm^{-2} actually required for inactivation of the triactinomyxons. The design of the CID ensures that organisms trapped temporarily on the screens before passing through the machine receive a minimum of 4000 mWs cm^{-2} and a maximum of 8000 mWs cm^{-2}.

For testing of the CID with parasite challenge studies, 13 rainbow trout (2.4 g) were placed in 2 l of water in each of the 6 tanks supplied with water treated by the CID. Water supply to the tanks was arrested for 5 min after the addition of the parasites to the CID to provide time for passage of the parasites through the machine; then water flow to each tank was resumed at a rate of 3 l min^{-1}. Parasites were introduced into the CID over a 12 min period. A period of 19 min was required to bring the volume to capacity in the 130 l tanks. Water flow into the tanks was stopped when full, and tanks were held with aeration but without water flow for 14 h to maximize contact between parasites in the aquaria. Flows were resumed the next day, and fish were maintained as previously described. Untreated triactinomyxon control exposures were designed to mimic conditions of the CID treated exposures. Thirteen fish were placed in 2 l of water in similar tanks, with water inflow set at 3 l min^{-1}. An amount of parasites equal to $\frac{1}{6}$ of that injected into the CID unit was added to each tank. Water flow was stopped when tanks were full. Tanks were held static overnight, with flows returned to 3 l min^{-1} to each tank the next day. A total of 64 200 triactinomyxons per fish was added through a series of 4 exposures over a 2 wk period to fish in both the CID and untreated water groups. A group of fish held under the same conditions but receiving only well water and no parasites served as unexposed controls. The well water temperature throughout the trial was maintained at 15°C. The study was conducted in the winter such that water temperatures would not increase or decrease significantly during static water periods of exposure. The fish were maintained as described previously (Andree et al. 1998). Five fish from replicate unexposed, CID exposed, and exposed untreated aquaria were examined by PCR analysis at 5 wk post-exposure (Andree et al.

1998). In addition, 5 fish from replicate unexposed, exposed untreated aquaria and 10 fish from each CID exposed aquaria were examined for cranial spore concentrations and microscopic lesions at 5 mo post-exposure. Two separate trials (Trials 1 and 2) were conducted following this procedure with the only difference that fish in Trial 1 received a cumulative dose over a period of 2 wk of 7539 triactinomyxons compared to 64 200 triactinomyxons per fish in Trial 2.

UV dose response curves for *Flavobacterium psychrophilum*. An additional study conducted with the UV collimating beam apparatus examined the inactivation of the bacterial pathogen *Flavobacterium psychrophilum*. The bacterium was grown in broth (Anacker & Ordal 1955) at 15°C for 72 h. Broth cultures were then distributed into 3 aliquots of 20 ml in sterile petri dishes. Two replicates were exposed to UV irradiation (140 µW cm^{-2}) and 1 dish was not irradiated (control). Aliqouts of 0.5 ml were removed at selected exposure times from all 3 plates, suspended in Anacker and Ordal broth for serial dilutions and spread onto tryptone yeast extract (TYES) agar for enumeration of viable cells. Colonies were counted after 7 d incubation at 15°C.

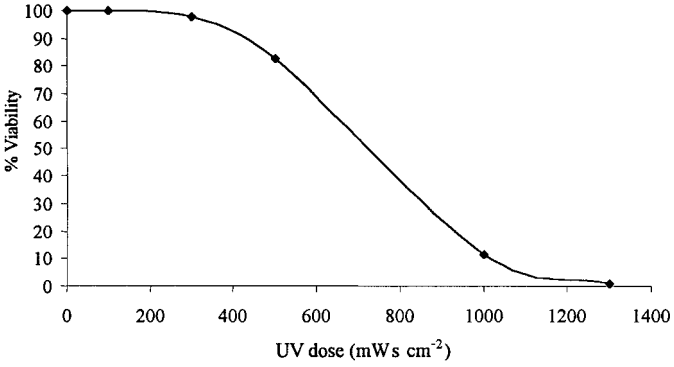


Fig. 1. Effects of cumulative dose of ultraviolet light on the viability of triactinomyxon stages of *Myxobolus cerebralis* as determined by vital staining

RESULTS

UV dose response curves—vital staining

A cumulative dose of 1300 mWs cm^{-2} was required to inactivate the triactinomyxons as suspended in well water under the conditions tested (Fig. 1). The sporoplasm cells in the apical end of the triactinomyxon all stained red after treatment at this dosage, suggesting that these cells were no longer viable. In contrast, 57% of the untreated triactinomyxons held under the same conditions contained sporoplasm cells all of which stained green, suggesting they were viable. Using the cumulative dose of 1300 mWs cm^{-2} as an estimate, trials with larger numbers of triactinomyxons were conducted for the subsequent fish exposure studies.

Effect of static (UV collimating beam) treatments on infectivity for trout

At 5 wk post-exposure and prior to the onset of clinical signs, all 10 fish receiving the high dose of untreated triactinomyxons were positive, while those fish receiving the UV-treated triactinomyxons were all negative (PCR test). In addition, 10 unexposed, untreated control fish were also negative. Clinical signs including black tail and whirling swimming first became evident at 6 wk following exposure to triactinomyxons in the high dose not treated with UV; by 2 mo 100% of the fish in the high dose group and 13% in the low dose group displayed clinical signs (Table 1). No clinical signs were observed among fish in any groups receiving UV-treated triactinomyxons. At 5 mo post-exposure, none of the trout receiving either the high or low dose triactinomyxons treated with UV had microscopic lesions or spores (Table 1). In contrast, 100% of the fish exposed to triactinomyxons not treated with UV

Table 1. Effects of static ultraviolet light treatments with the collimating beam apparatus on the infectivity of 2 concentrations of triactinomyxons (High and Low) for juvenile rainbow trout. nd: no spores detected

	Triactinomyxon exposure				
	UV-treated		No treatment		
Parasite exposure dose[a]:	High	Low	High	Low	None
Presence of clinical signs[b]	0/64	0/69	54/54	8/62	0/66
Prevalence of infection[c]	0/20	0/20	20/20	20/20	0/20
Mean lesion score[d]	0	0	3.0	2.2	0
Mean spore count[e]	nd	nd	$10^{5.6}$	$10^{4.6}$	0

[a]Dose of triactinomyxon stages of *Myxobolus cerebralis* per fish used in the exposures for High was 14 000 and Low 1400 triactinomyxons per fish

[b]Presence of black tail or whirling swimming at 2 mo post-exposure

[c]Infection with *M. cerebralis* was determined by presence of spores in the pepsin trypsin digestion of ½ of the head of 20 individual fish (10 from each of 2 replicate groups per treatment) at 5 mo post-exposure

[d]Microscopic lesions were scored on a scale of none (0) to severe (5) among 10 fish in the high exposure group at 5 mo post-exposure

[e]Mean concentration of spores (per ½ head) among infected fish in each group at 5 mo post-exposure

had microscopic lesions in the cranium and gills, with mean overall severity scores of 3.0 (high triactinomyxon exposure) and 2.2 (low triactinomyxon exposure). Mean spore concentrations for fish in the high and low group exposure to untreated triactinomyxons were $10^{5.6}$ and $10^{4.6}$ per ½ head, respectively (Table 1).

Efficacy of CID UV treatments on infectivity for trout

Two trials conducted with the CID provided similar results demonstrating the efficacy of this water treatment in eliminating infectivity of the triactinomyxon stage of *Myxobolus cerebralis* from water supplied to juvenile trout. At 5 wk post-exposure, prevalence of PCR-positive fish in groups receiving untreated triactinomyxons was less in Trial 1 (1/6) than in Trial 2 (6/6). In both trials fish receiving CID-treated triactinomyxons were PCR-negative (0/6). In addition, 6 unexposed, untreated control fish in both Trials 1 and 2 were also PCR-negative. But for 1 fish in the untreated triactinomyxon-exposed groups in Trial 2, no clinical signs were observed during the course of either trial. In both trials at 5 mo post-exposure, none of the 20 fish sampled from the CID-treated groups had microscopic lesions or spores (Table 2). In contrast, 100 % of the fish receiving untreated triactinomyxons contained microscopic lesions in the cranium and gills, with a mean score of 2.4 in Trial 2. No microscopic pathological examinations were conducted in Trial 1. The mean spore concentrations per ½ head for the untreated triactinomyxon group was greater in Trial 2 than in Trial 1.

Table 2. Efficacy of the *Cryptosporidium* Inactivation Device (CID) in protecting juvenile rainbow from exposures to triactinomyxons of *Myxobolus cerebralis*. nd: no spores detected

	Triactinomyxon-exposed[a]				Unexposed controls	
	CID-treated		No treatment			
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Prevalence of infection[b]	0/20	0/20	18/20	20/20	0/20	0/20
Mean lesion score[c]	–	0	–	2.4	0/20	0/20
Mean spore count[d]	nd	nd	$10^{4.4}$	$10^{4.6}$	nd	nd

[a]Fish were exposed to a cumulative dose of 7539 (Trial 1) or 64 200 (Trial 2) triactinomyxons per fish over a 2 wk period

[b]Infection with *M. cerebralis* was determined by presence of spores in the pepsin trypsin digestion of ½ of the head of 20 individual fish (10 from each of 2 replicate groups per treatment) at 5 mo post-exposure

[c]Microscopic lesions were scored on a scale of none (0) to severe (5) among 10 fish in the high exposure group at 5 mo for Trial 2 only

[d]Mean concentration of spores among infected fish in each group at 5 mo post-exposure

Table 3. Effects of static ultraviolet light treatments with the collimating beam apparatus on the viability of *Flavobacterium psychrophilum*. The numbers of viable bacteria are expressed as colony forming units ml^{-1}

Time (min)	Untreated control	Treated with UV irradiation	
		Replicate 1	Replicate 2
0	2.2×10^5	1.0×10^5	6.0×10^4
5	2.6×10^5	1.0×10^5	5.0×10^4
15	2.3×10^5	0	0
30	2.2×10^5	0	0

UV dose response curves for *Flavobacterium psychrophilum*

Treatments of suspensions of *Flavobacterium psychrophilum* with UV irradiation were ineffective at doses of 42 mW s cm^{-2} (Table 3). However, at doses of 126 or 252 mW s cm^{-2} bacterial cells were killed by the treatment (Table 3).

DISCUSSION

UV irradiation was effective in destroying the viability of the triactinomyxon stages of *Myxobolus cerebralis* as determined by both vital staining and elimination of infectivity for young rainbow trout. UV treatments were highly effective whether administered in small volumes with a low pressure collimating beam apparatus, or in larger volumes with higher intensity radiation in a flow-through device (CID) designed for water supplies. These results demonstrated the feasibility of UV irradiation, even at larger scales needed for hatchery water supplies, to prevent infections of hatchery reared trout with *M. cerebralis*. In addition, the UV dose effective against *M. cerebralis* also inactivated the bacterium *Flavobacterium psychrophilum*, the cause of salmonid bacterial coldwater disease.

UV irradiation has been most commonly used to eliminate or reduce pathogens in closed recirculating fish rearing units but also in larger scale fish production units using single-pass water (Hoffman 1974). The principal mode of action for UV irradiation, particularly that in the 220 to 300 nm wavelengths, is the formation of pyrimidine dimers in the target cell DNA

(Schechmeister 1991). Among the many stages of *Myxobolus cerebralis*, the most susceptible form to both chemical and physical destruction is the triactinomyxon, the form released into the water from the oligochaete host (Markiw 1992b). This is the form present in the water that effectively attaches to the epidermis of the fish and then proceeds to invade through the skin, migrating to the nerves, and finally to the cartilage (Markiw 1989, El-Matbouli et al. 1995). This stage of the parasite is large (up to 180 µm between valve tips) and possesses relatively thin valves as protection for the approximately 64 sporoplasm cells that are packaged for delivery into the fish epidermis (El-Matbouli et al. 1999). Although unknown at the time, the first trials with UV with *M. cerebralis* by Hoffman (1974, 1975) were targeting these triactinomyxon stages. Hoffman (1974, 1975) recommended treatments of 35.0 mW s cm^{-2} for the elimination of *M. cerebralis* infectivity from water supplies. He found that treatments of 18.0 to 27.7 mW s cm^{-2} gave less consistent results, protecting fish from clinical disease but not all fish from infections.

In our trials with the collimated beam apparatus, levels of 1300 mW s cm^{-2} were required to inactivate triactinomyxons as judged by the lack of viable staining of all of the sporoplasm cells in all of the triactinomyxons. This dosage is probably in excess of that required as it may not be necessary to inactivate all the sporoplasm cells to render the triactinomyxon noninfectious. This in part may explain why much lower doses were effective in the trials by Hofmann (1974, 1975).

Treatments of larger volumes of water in hatchery water supplies, particularly during early fish rearing, is viewed as one possible means to aid in the control of whirling disease. While the flow rates examined in our study were below the capacity of the apparatus, the CID effectively eliminated infectivity of triactinomyxons as administered in repeated dosages over a 2 wk period in 2 separate trials. In the CID, water passing through the 2 µm screen receives a dose of 4000 mW s cm^{-2} and materials temporarily trapped on the filter can be treated at even higher doses up to 8000 mW s cm^{-2}. These doses of UV irradiation are well above the 1300 mW s cm^{-2} determined from the static trials to eliminate all infectivity due to *Myxobolus cerebralis*. We presume, but did not test, that the physical forces during the temporary filtration experienced by triactinomyxons entering the CID could contribute to shearing of the parasite that might also greatly reduce infectivity. We have found that although triactinomyxons are large when fully extended (up to 180 µm) they can fold upon themselves and effectively pass through screen sizes of 20 µm (authors' unpubl. data). However, triactinomyxons entering a 2 µm screen under pressure would be retained and only sporoplasm stages, which presumably are noninfectious once released from the surrounding membrane, would pass the screen (El-Matbouli et al. 1999). Larger models of the CIDs are available with capabilities to treat up to 3344 l min^{-1}, and these could be used for early rearing of salmonids to prevent serious infections with *M. cerebralis*. At one local hatchery, we estimated that 2 larger CID would be sufficient to rear 200 000 steelhead trout through the first 4 mo after hatching.

UV irradiation was also effective in destroying the salmonid bacterial pathogen *Flavobacterium psychrophilum*, a pathogen often occurring in the same hatcheries as whirling disease. Furthermore, this bacterium is prevalent among salmonid fish throughout the world and a major cause of losses among hatchery-reared rainbow trout (Bernardet et al. 1996). The bacterium is transmitted through the water column from fish to fish and presumably with eggs from infected adult fish (Brown et al. 1997). The bacterium as shed into the water would pass the 2 µm screen in the CID but the 4000 mW s cm^{-2} dose experienced as it passed the filter is well in excess of the 126 mW s cm^{-2} dose shown to inactivate over 10^5 colony forming units of the bacterium in our trials (Table 3).

Certain mechanisms for the transmission of whirling disease remain unknown, but stocking or transfer of live infected fish to new locations is clearly one known mode (Modin 1998). When the suitable oligochaete and fish hosts and environment are present, whirling disease can thrive. If these conditions are not met, the disease may fail to be established or remain at a low level that in part may explain the differential impacts of whirling disease in the intermountain west compared to other eastern and western states (Hedrick et al. 1998). Groundwater should be used whenever possible for hatchery-rearing of salmonids in whirling disease enzootic waters (Hoffman 1990), but when this is not possible, treatment of the incoming surface water with UV is a feasible alternative to prevent infection with *Myxobolus cerebralis*. Our controlled laboratory trials and those of Hoffman (1974, 1975) demonstrate that UV irradiation, particularly when delivered by newly designed and more efficient apparatuses, should be seriously considered for hatchery programs aimed at stocking fish when surface waters contain the infectious stages of *M. cerebralis*.

Acknowledgements. Funding for this research came from the Colorado Division of Wildlife and Safe Water Solutions L.L.C. Contributors to this work included Mr Mark Sampson of Water Control Corporation and Mr Chuck Reading of Safe Water Solutions L.L.C. and Clancy Environmental Consultants Inc.

LITERATURE CITED

Anacker RL, Ordal EJ (1955) Study of a bacteriophage infecting myxobacterium *Chondrococcus columnaris*. J Bacteriol 70:738–741

Andree KB, MacConnell E, Hedrick RP (1998) A nested polymerase chain reaction for the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 34:145–154

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). J Syst Bacteriol 46:128–148

Brown LL, Cox WT, Levine RP (1997) Evidence that the causal agent of bacterial coldwater disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Dis Aquat Org 29:213–218

Bukhari Z, Hargy TM, Bolton JR, Dussert B, Clancy JL (1999) Inactivation of *Cryptosporidium parvum* oocysts using medium-pressure ultraviolet light. J Am Water Works Assoc 91:86–94

Campbell AT, Robertson LJ, Snowball MR, Smith HV (1995) Inactivation of oocysts of *Cryptosporidium parvum* by ultraviolet irradiation. Water Res 29:2583–2586

Clancy JL, Hargy TM, Marshall MM, Dyksen JE (1998) Inactivation of *Cryptosporidium parvum* oocysts in water using ultraviolet light. J Am Water Works Assoc 90: 92–102

El-Matbouli M, Fischer-Scherl T, Hoffman RW (1992) Present knowledge of the life cycle, taxonomy, pathology, and therapy of some *Myxosporea* spp. important for freshwater fish. Annu Rev Fish Dis 3:367–402

El-Matbouli M, Hoffmann RW, Mandok C (1995) Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of *Myxobolus cerebralis* from epidermis into rainbow trout (*Oncorhynchus mykiss*) cartilage. J Fish Biol 46:919–935

El-Matbouli M, Hoffmann RW, Shoel H, McDowell TS, Hedrick RP (1999) Whirling disease: host specificity and interaction between the actinosporean stage of *Myxobolus cerebralis* and rainbow trout (*Oncorhynchus mykiss*) cartilage. Dis Aquat Org 35:1–12

Halliday MM (1976) The biology of *Myxosoma cerebralis*; the causative agent of whirling disease of salmonids. J Fish Biol 9:339–357

Hedrick RP, El-Matbouli M, Adkison MA, MacConnell E (1998) Whirling disease: re-emergence among wild trout. Immunol Rev 166:365–376

Hedrick RP, McDowell TS, Mukkatira K, Georgiadis MP, MacConnell M (1999a) Susceptibility of selected inland salmonids to experimentally-induced infections with *Myxobolus cerebralis* the causative agent of whirling disease. J Aquat Anim Health 11:330–339

Hedrick RP, McDowell TS, Gay M, Marty GD, Georgiadis MP, MacConnell M (1999b) Comparative susceptibility of rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the cause of salmonid whirling disease. Dis Aquat Org 37:173–183

Hofer B (1903) Über die Drehkrankheit der Regenbogenforelle. Allg Fisch-Ztg Jahrg XXVIII (1):7–8

Hoffman GL (1970) Intercontinental and transcontinental dissemination and transfaunation of fish parasites with emphasis on whirling disease (*Myxosoma cerebralis*) In: Snieszko SF (ed) A symposium on diseases of fishes and shellfishes. Am Fish Soc, Washington, DC, p 69–81

Hoffman GL (1974) Disinfection of contaminated water by ultraviolet irradiation, with and emphasis on whirling disease (*Myxosoma cerebralis*) and its effect on fish. Trans Am Fish Soc 103:541–550

Hoffman GL (1975) Whirling disease (*Myxosoma cerebralis*) control with ultraviolet irradiation and effect on fish. J Wildl Dis 11:505–507

Hoffman GL (1990) *Myxobolus cerebralis*, a worldwide cause of salmonid whirling disease. J Aquat Anim Health 2: 30–37

Markiw ME (1989) Portals of entry for salmonid whirling disease in rainbow trout. Dis Aquat Org 6:7–10

Markiw ME (1992a) Salmonid whirling disease. U.S. Department of Interior, Fish and Wildlife Service, Fish and Wildlife Leaflet 17, Washington, DC, p 1–11

Markiw ME (1992b) Experimentally-induced whirling disease. II. Determination of longevity of the infective triactinomyxon stage of *Myxobolus cerebralis* by vital staining. J Aquat Anim Health 4:44–47

Modin J (1998) Whirling disease in California: a review of its history, distribution, and impacts, 1965–1997. J Aquat Anim Health 10:132–142

Nehring RB, Walker PG (1996) Whirling disease in the wild: the new reality in the intermountain west. Fisheries (Bethesda) 21:28–32

Schaperclaus W (1986) Fish diseases, Vol 2, IV Protoziases. Akademie-Verlag, Berlin, p 665–666

Schechmeister II (1991) Sterilization by ultraviolet irradiation. In: Block SS (ed) Disinfection, sterilization, and preservation, 4th edn. Lea and Febiger, Philadelphia, PA, p 553–565

Thoesen JC (1994) Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 4th rev edn, Ver 1. Fish Health Section, American Fish Society, Bethesda

Vincent ER (1996) Whirling disease and wild trout: the Montana experience. Fisheries (Bethesda) 21:32–34

Wagner E (1998) TAM vital staining: effect of sodium hypochlorite on viability. The Ichthyogram 9(4):4–5

Wolf K (1986) Salmonid whirling disease: status in the United States, 1985. J Wildl Dis 22:295–299

Wolf K, Markiw ME (1984) Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452

Editorial responsibility: Wolfgang Körting, Hannover, Germany

Submitted: February 15, 2000; Accepted: May 8, 2000
Proofs received from author(s): July 11, 2000

Vol. 6: 295–298, 1981 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published November 15

Pathogen Reduction in Closed Aquaculture Systems by UV Radiation: Fact or Artifact?*

Stephen Spotte[1] and Gary Adams[2]

[1] Sea Research Foundation, Inc., Mystic Marinelife Aquarium, Mystic, Connecticut 06355, USA
[2] Department of Mathematics and Physics, Thames Valley State Technical College, Norwich, Connecticut 06360, USA

ABSTRACT: Differential equations were used to set a theoretical upper limit for the efficacy of UV radiation in 3 hypothetical aquaculture systems: (a) a plug-flow system, (b) an idealized closed system with no influx of pathogens, and (c) a conventional closed system in which the influx of pathogens is continuous. The equations demonstrate that, in a conventional closed system, the mass of pathogens never reaches zero even if the UV sterilizer is 100 % effective. This suggests that agents such as UV radiation, which do not form persistent residuals, may be incapable of preventing the spread of waterborne pathogens in systems that are recirculated. Use of UV radiation in aquaculture is most effective in sterilization of raw water supplies and discharges into receiving waters, both of which are single-pass applications.

INTRODUCTION

Sterilizing agents commonly are used in aquaculture installations, fish hatcheries, and public aquariums, in which crowded conditions often hasten the spread of transmissible diseases. Diseases produced by single-celled organisms, or multicellular organisms that do not require vectors, are particularly troublesome in closed systems, because all water is reused, rather than diluted continuously with clean influent. Free-floating infectious organisms (referred to here simply as pathogens) accumulate, unless the sterilization rate equals or exceeds the rate of contamination.

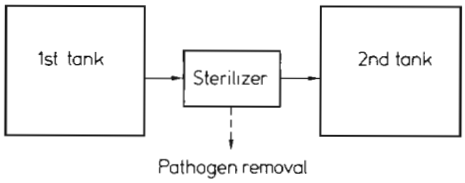
METHOD

We used differential equations to model the performance of UV sterilization in 3 hypothetical water systems illustrated in Fig. 1. The curves in Fig. 2 show the mass of pathogens remaining in the systems after sterilization. The effective kill rate on contact in the sterilizer was presumed to be 100 % for each of the 3 schemes. Scheme A of Fig. 1 and Curve A of Fig. 2 show the total mass of pathogens remaining if water flows through the sterilizer in a single pass into a second tank with no replacement water (plug-flow system). Scheme B of Fig. 1 and Curve B of Fig. 2 illustrate a system in which sterile water is returned from the UV unit to the culture facility with complete mixing, but without the influx of additional pathogens (idealized closed system). Scheme C of Fig. 1 and Curve C of Fig. 2 represent a conventional closed system; that is, water returned from the sterilizer is disease-free, but the influx of pathogens from within the culture facility itself is constant. The percent kill of pathogens in Scheme C is limited by an equilibrium level at which the kill rate equals the rate of influx. This equilibrium equals the rate of influx divided by the flow rate through the sterilizer. The efficacy of the sterilizer depends on only 2 factors: the rate at which pathogens are introduced and the effectiveness of the treatment device, as shown in the equations below. The same equations can be used to calculate the rate at which chemical contaminants are removed by physical adsorption contactors, such as foam fractionators and activated carbon beds.

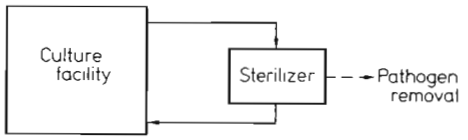
Symbols: V = total volume of the system (constant); C_o = initial mass of pathogens (constant); t_o = initial time; F = volume flow rate through the sterilizer (constant); C = mass of pathogens; $\frac{FC}{V}$ = kill rate (mass per unit time); R = influx of pathogens (mass per unit time)

* Contribution No. 22, Sea Research Foundation, Inc.

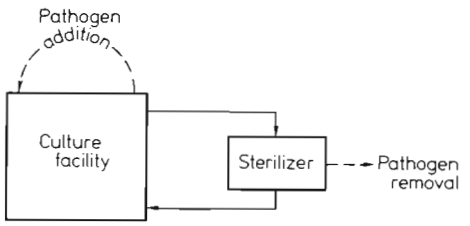
0171-8630/81/0006/0295/$ 02.00



Scheme A



Scheme B



Scheme C

Fig. 1. Plug-flow system (Scheme A): water flows through the sterilizer in a single pass, and pathogens are removed at a constant rate. Idealized closed system (Scheme B): all water is recycled, there is no new addition of pathogens, and organisms are removed at a rate proportional to concentration. Conventional closed system (Scheme C): all water is recycled, pathogens are added at a constant rate, and removed at a rate proportional to concentration. The rate of sterilization at all 3 contact sites is assumed to be 100 %

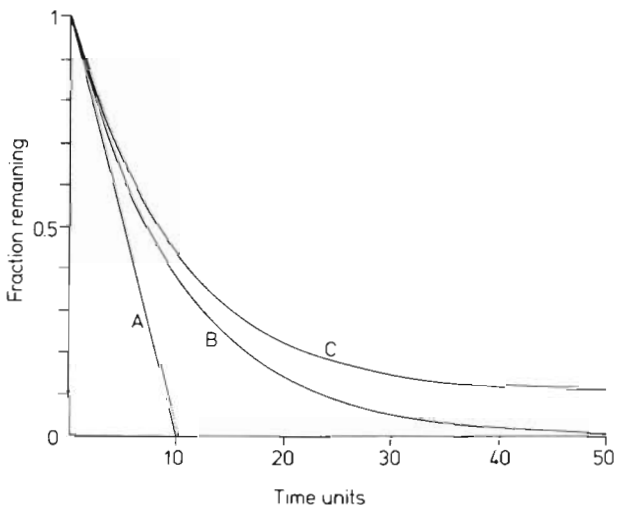


Fig. 2. Mass of pathogens remaining in Schemes A through C (Fig. 1) versus time. In closed systems (Scheme C), complete sterilization is unattainable

Scheme A

In Scheme A, water is pumped through the sterilizer on a single pass and discharged into a second tank. No water is recycled. If F = 0.1 V (10 % of the system volume h^{-1}), it would take 9 h to kill 90 % of the pathogens in the system.

Scheme B

In Scheme B, all water is recycled through the sterilizer and returned continuously to the main portion of the system. There is no new influx of pathogens. Thus

$$dC = -C\left(\frac{F}{V}\right)dt$$

$$\frac{dC}{C} = -\left(\frac{F}{V}\right)dt$$

$$\ln C = \frac{-F}{V}t + k$$

$$\text{at } t_o = 0,\ C = C_o$$

$$\ln C_o = k$$

Therefore

$$\ln \frac{C}{C_o} = -\frac{Ft}{V}$$

and

$$C = C_o e^{-\frac{Ft}{V}}$$

If F = 0.1 V per unit time

$$C = C_o e^{-0.1t}$$

and

$$C = 0.1\ C_o \text{ (90\% kill)}$$

$$e^{-0.1t} = 0.1$$

then

$$t = 23 \text{ time units}$$

For a flow through the sterilizer equal to 10 % of the volume of the system per hour, 23 h are required to kill 90 % of the pathogens.

Scheme C

In Scheme C, all water is recycled through the sterilizer and returned continuously to the original source, as in Scheme B. However, there also is a continuous influx of pathogens at rate R, and

$$dC = -C\left(\frac{F}{V}\right)dt + Rdt$$

$$dC = \left[-C\left(\frac{F}{V}\right) + R\right]dt$$

Integration yields

$$C = e^{\frac{-Ft}{V}}\left(C_o - \frac{RV}{F}\right) + \frac{RV}{F}$$

If F = 0.1 V and R = 0.01 for a 90% kill, C = 0.1 C_o, then because

$$C = e^{\frac{-Ft}{V}}\left(C_o - \frac{RV}{F}\right) + \frac{RV}{F}$$

in this case

$$C = 0.9\ C_o e^{-0.1t} + 0.1$$

By substituting different values for t it can be shown that a 90% kill can be attained only after infinite time. In fact, the mass of pathogens remaining cannot be less than $\frac{RV}{F}$, which in this case is 10% of C_o.

DISCUSSION

As noted by Herald et al. (1970), the effectiveness of UV radiation is limited to *in vitro* situations; in other words, to the destruction of pathogens that are free-floating in the water. Organisms that are systemic, or attached to exterior surfaces of their hosts, are unaffected and can only be controlled chemotherapeutically. Organisms shed into the water, or which detach from their hosts, thus represent a reservoir of contamination that cannot be eliminated from closed systems, if sterilization occurs at a single contact site (Scheme C).

Spotte (1979) reviewed the use of UV radiation in aquatic animal culture. Some pathogens always survive, despite kill rates that sometimes approach 100 % at the contact site. Animals maintained in closed systems thus are subject to possible reinfection from water returning from the sterilizer, the degree of reinfection depending on the virulence and concentration of the pathogen and the immune status of the host. Bullock and Stuckey (1977) studied the effect of UV radiation on bacterial counts of salmonid hatchery water. In some instances, bacteria at the contact site were reduced 99.99 %, but the authors cautioned against placing undue emphasis on the results, because the number of bacteria necessary to transmit disease is difficult to predict. They pointed out that, in their experiments, a 99.99 % kill of a pathogen at a cell density of 10^4 ml^{-1} would leave only 1.0 ml^{-1}. They concluded that even this low concentration might be adequate to transmit disease during intensive culture if the pathogen is virulent, considering the growth potential of bacteria. Buck (1980) recorded low cell densities of a variety of potentially pathogenic fungi in a UV-treated marine mammal pool operated as a closed system. Spotte and Buck (1981) studied the efficacy of UV sterilization on pathogen reduction in the same pool. They observed that cutaneous and systemic infections in the captive dolphins and whales, caused by the yeast *Candida albicans*, continued to flourish even though cell counts never exceeded 100 l^{-1} anywhere in the water system.

Disease-causing organisms can also be transmitted among cultured animals directly, short-circuiting the sterilizer completely (Scheme C). The result may be a high percent kill at the contact site that is not accompanied by a concomitant decrease in the incidence of disease or mortality. For example, Herald et al. (1970) reported that mortality rates among exhibit fishes at a public aquarium were unchanged by installation of a UV sterilizer that lowered the total bacteria at the contact site by 98 %. Spanier (1978) noted that mortality rates of bream *(Sparus aurata)* larvae were unaffected by UV sterilization of the recycled water, despite a substantial decrease in bacterial counts at the sterilizer effluent.

The application of UV radiation in the treatment of raw influent water is effective in lowering the numbers of pathogens and thus reducting the chances of disease organisms entering from external sources (Stickney and White, 1974; Hoffmann, 1975; Kimura et al., 1976; Blogoslawski et al., 1978; Brown and Russo, 1979). Results derived from this application can be considered f a c t. If the water is recycled, however, any apparent efficacy of a UV sterilizer, based on kill rate, is an a r t i f a c t, because the mass of pathogens in the immediate vicinity of the cultured animals is always greater than the mass in the sterilizer effluent. Equilibrium is never attained, and the entire system cannot be rendered disease-free, even when the sterilization process is 100 % effective.

In conclusion, UV sterilization should perhaps be limited to the treatment of influent water supplies, or to the final effluent from culture installations before it is discharged into receiving waters. Both are single-pass applications, for which the effectiveness of UV treatment is well documented.

Acknowledgements. We wish to thank James W. Atz, Carol E. Bower, Walter J. Blogoslawski, John B. Gratzek, and Gary D. Pruder for their reviews of the manuscript.

LITERATURE CITED

Blogoslawski, W. J., Stewart, M. E., Rhodes, E. W (1978). Bacterial disinfection in shellfish hatchery disease control. Proc. World Maricult. Soc. 9: 589–602

Brown, C., Russo, D. J. (1979). Ultraviolet light disinfection of shellfish hatchery sea water. I. Elimination of five pathogenic bacteria. Aquaculture 17: 17–23

Buck, J. D. (1980). Occurrence of human-associated yeasts in the feces and pool waters of captive bottlenosed dolphins *(Tursiops truncatus)*. J. Wildl. Dis. 16: 141–149

Bullock, G. L., Stuckey, H. M. (1977). Ultraviolet treatment of water for destruction of five gram-negative bacteria pathogenic to fishes. J. Fish. Res. Bd Can. 34: 1244–1249

Herald, E. S., Dempster, R. P., Hunt, M. (1970). Ultraviolet sterilization of aquarium water. In: Hagen, W. (ed.) Aquarium design criteria. (Spec. ed.), Drum and Croaker, U. S. Department of the Interior, Washington, DC, pp. 57–71

Hoffman, G. L. (1975). Whirling disease *(Myxosoma cerebralis)*: Control with ultraviolet irradiation and effect on fish. J. Wildl. Dis. 11: 505–507

Kimura, T., Yoshimizu, M., Tajima, K., Ezura, Y., Sakai, M. (1976). Disinfection of hatchery water supply by ultraviolet (U. V.) irradiation. I. Susceptibility of some fish-pathogenic bacterium and microorganisms inhabiting pond waters. Bull. Jap. Soc. Sci. Fish. 42: 207–211

Spanier, E. (1978). Preliminary trials with an ultraviolet liquid sterilizer. Aquaculture 14: 75–84

Spotte, S. (1979). Seawater aquariums: The captive environment, Wiley, New York

Spotte, S., Buck, J. D. (1981). The efficacy of UV irradiation in the microbial disinfection of marine mammal water J. Wildl. Dis. 17: 11–16

Stickney, R. R., White, D. B. (1974). Lymphocystis in tank-cultured flounder. Aquaculture 4: 307–308

This paper was submitted to the editor; it was accepted for printing on July 23, 1981

Aquacultural Engineering 55 (2013) 9–15

EXHIBIT E-8

Contents lists available at SciVerse ScienceDirect

Aquacultural Engineering

journal homepage: www.elsevier.com/locate/aqua-online

Prevention of fungal infestation of rainbow trout (*Oncorhynchus mykiss*) eggs using UV irradiation of the hatching water

J. Heikkinen[a,*], S.M. Mustonen[b], P. Eskelinen[c], L.-R. Sundberg[d], A. Von Wright[a,1]

[a] Department of Biosciences, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland
[b] Department of Environmental Science, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland
[c] Finnish Game and Fisheries Research Institute, Jyväskylä Game and Fisheries Research, Survontie 9, FI-40500 Jyväskylä, Finland
[d] Centre of Excellence in Biological Interactions, Department of Biological and Environmental Science, University of Jyväskylä, PO Box 35, FI-40014 Jyväskylä, Finland

ARTICLE INFO

Article history:
Received 19 July 2012
Accepted 24 January 2013

Keywords:
UV irradiation
Ozone
Hydrogen peroxide
Formalin
Saprolegnia

ABSTRACT

Fungal infestation by water mold *Saprolegnia* spp. causes great losses in aquaculture and fish egg hatching. To find a safe and effective alternative for the fungal prevention, we studied continuous disinfection of the inlet water by UV irradiation and ozonation combined with low concentration hydrogen peroxide (H_2O_2) treatments in a rainbow trout (*Oncorhynchus mykiss*) egg hatching system. High dose of UV irradiation (400 mWs/cm^2) of the inlet water decreased the mortality of rainbow trout eggs from the 77.3% to 14.3% in a 28 day trial. UV irradiation did not modify water quality parameters, while combination of UV irradiation and H_2O_2 caused up to fivefold increase in the formate levels, and combination of O_3 and H_2O_2 caused even ten-fold increase in the acetate and formate levels. UV suppressed the gradual increase of the heterotrophic bacterial counts on the fish eggs. Based on the molecular profiling high dose of UV reduced the growth of some of the dominating bacterial groups and combination of UV and H_2O_2 had a distinctive effect on the overall bacterial community structure on the fish eggs.

1. Introduction

Infections of ubiquitous Oomycete species, *Saprolegnia* sp., cause annually great losses in production of different freshwater fish and fish eggs (Hussein et al., 2001; Fregeneda-Grandes et al., 2007; Thoen et al., 2011). Malachite green was effective treatment against *Saprolegnia* infections in fish eggs and adults, but after it was banned in 1991 in United States (Marking et al., 1994) and 2001 in European Union, research for substitutive treatments have been intensive (Marking et al., 1994; Schreier et al., 1996; Pottinger and Day, 1999; Forneris et al., 2003; Straus et al., 2012; Caruana et al., 2012). Although several potential replacements have been suggested, effective, safe, ecologically and economically acceptable methods have not been found yet (van West, 2006).

Formalin has been successfully used in disinfection of *Saprolegnia*-infected fish (Gieseker et al., 2006) and fish eggs (Schreier et al., 1996; Barnes et al., 2000; Rach et al., 2005) being the mostly used treatment in fish farms. However, safety and environmental concerns are related to the use of formalin in hatcheries (Marking et al., 1994). Formalin fumes causes irritation in eyes and respiratory ducts in humans, therefore sufficient ventilation and awareness of the risks are needed. Levels used in aquaculture have not been found to cause occupational health hazards (Lee and Radtke, 1998; Wooster et al., 2005).

Hydrogen peroxide is reported to effectively control saprolegniasis of fish eggs (Marking et al., 1994; Waterstrat and Marking, 1995; Schreier et al., 1996; Rach et al., 1998; Arndt et al., 2001) at concentrations 500 mg/l or greater. Arndt et al. (2001) reported increased survival in rainbow trout egg incubation, when daily 500 ppm hydrogen peroxide treatment was withheld during days 7–11 after fertilization suggesting greater toxicity of H_2O_2 for eggs during this sensitive period. Furthermore, hydrogen peroxide treatments should be avoided near hatching (Wagner et al., 2012). Hydrogen peroxide is also used along with ozone or UV irradiation in advanced oxidation processes (AOP), but effective levels of H_2O_2 with UV-irradiation were 23 mg/l or higher for effective production of $^{\bullet}$OH radicals (Rosenfeldt et al., 2006; Toor and Mohseni, 2007).

High bacterial levels on egg surface may attract opportunistic fish pathogens and hence impede egg development (Barker et al., 1989, 1991; Barnes et al., 2005). Decreasing the bacterial levels and destroying *Saprolegnia* spores from the inlet water has therefore been suggested for prevention of fungal infections of rainbow trout eggs (Barker et al., 1990). UV irradiation and ozonation treatments are the most used methods in aquaculture for disinfecting

* Corresponding author. Present address: Department of Biology, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland. Tel.: +358 403553373.
E-mail address: jouni.heikkinen@uef.fi (J. Heikkinen).
[1] Present address: Institute of Public Health and Clinical Nutrition, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland.

0144-8609/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaeng.2013.01.001

the inlet water. UV irradiation is strongly germicidal and does not produce harmful by-products (Wolfe, 1990) and it has therefore been used in aquaculture for reduction of total aerobic bacterial numbers and fish pathogens in the inlet water (Brown and Russo, 1979; Hedrick et al., 2000; Sharrer et al., 2005). Although UV-irradiation is widely used in drinking water technology and fish farms, especially in recirculation aquaculture, only few studies have reported its applications in flow-through aquaculture systems. It is known that organic particles decrease the efficacy of UV disinfection of particle-associated bacteria (Emerick et al., 1999; Summerfelt, 2003). Hence the quality of treated water has a strong effect on the UV-based disinfection, and proper water filtration prior to UV treatment is required when the turbidity of the water is high.

Ozone has been widely used in drinking water technology (Von Gunten, 2003a). The main purposes of ozone use are disinfection and oxidation of organic matter for improving the water quality. It is also germicidally effective, and it has been used in aquaculture to inactivate fish pathogens, oxidize organic matter and nitrite, or to enhance effectiveness of other water treatment units, like UV-irradiation systems. As advantages, ozone has a fast reaction rate, it produces only few non-wanted reaction by-products in freshwater, and the end product (oxygen) decreases the need of additional aeration or oxygenation of the water. Use of ozonation in aquaculture has still limits due to its high expenses and the narrow safety margin between the effective and harmful doses (Forneris et al., 2003). Furthermore, several studies reveal multiple significant negative associations between ozone exposure and pulmonary function for human (Scannell et al., 1996; Bhalla, 1999; Blomberg et al., 1999; Klestadt et al., 2002) and for this reason the escape of ozone to the air must be prevented with e.g. ozone destructor. Although ozone generation and destruction equipments are expensive, the use of ozone has potential in aquaculture (Liltved et al., 1995; Summerfelt and Hochheimer, 1997; Forneris et al., 2003), especially in disinfection of recirculated water (Good et al., 2011; Davidson et al., 2011).

Although several anti-fungal treatment methods have shown positive effects on the egg survival and hatching rates in the fish farms, comprehensive studies comparing these methods and their possible combinations are still lacking. Three different disinfection methods of inlet water ($O_3 + H_2O_2$, UV, UV + H_2O_2) were therefore investigated in this study to find out whether these treatments would significantly decrease *Saprolegnia* sp. infection of rainbow trout eggs. Effects of the different treatments were further studied using cultivation and molecular studies of the bacterial epibiota, to evaluate the effects on the total aerobic bacterial counts and microbial diversity (number of operational taxonomical units, OTU's) hosted on the rainbow trout egg surface.

2. Materials and methods

2.1. Incubation trial

Rainbow trout egg hatching experiment was carried out in five groups (two control groups and three treatment groups) as triplicates. The first control group (1) did not get any anti-fungal treatments, but the second control group (2) simulated the normal procedure in rainbow trout hatchery, in which the eggs were treated with formalin baths (1000 mg/l concentration, 15 min/day, 5 times a week) after *Saprolegnia* sp. hyphae were visually detected on eggs. The inlet water of the three treatment groups was treated as follows: (3) $O_3 + H_2O_2$, (4) UV, (5) UV + H_2O_2 (Henceforth the treatments are discussed with same numerals). The doses (see chapters 2.2–2.4) were chosen according to the results of pre-trials (data not shown).

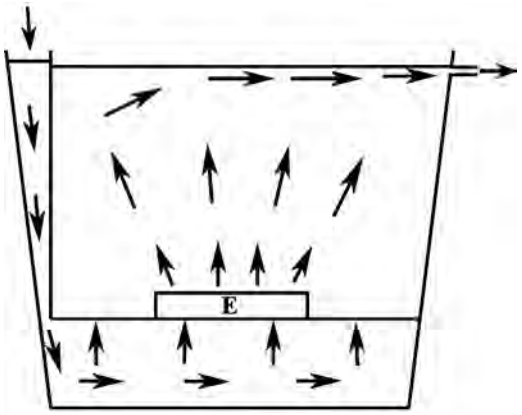


Fig. 1. Diagram of the water flow (shown by arrows) in the hatching tanks (volume 10 l) carrying the hatching tray (E).

Incubation experiments were carried out in 10 litre tanks in which smaller incubation trays were installed (Fig. 1). Rainbow trout (*Oncorhynchus mykiss*) eggs were collected from 105 females and fertilized with the milt of 6 sex-reversed female trouts (95% fertilization percentage), acquired from Savon Taimen fish farm, Rautalampi, Finland. The eggs were carefully poured on the trial trays (surface area approximately 200 cm^2, 0.1 litres eggs per tray, about 1300 eggs total). Dead eggs were not removed before the eyed-egg stage during the trial to reflect the prevailing incubation methods (upwelling incubators) used in Finland. Temperature of the water (Lake Kallavesi) used in the trial remained $10 \pm 0.6\,^{\circ}C$ during the experiment. Formalin baths on the control group 2 were started on day 8, when first *Saprolegnia* sp. infected eggs were visually detected. Eyed-egg stage was reached after 17 days and the trial was ended on day 28.

2.2. $O_3 + H_2O_2$

Ozone was produced from pressurized air using an ozone generator (O1, Pacific Ozone Technology, Benicia, California, USA). The system also included contact column (volume 17 l), an analyser for gaseous ozone (Dasibi 1180-HC, Dasibi Environmental Corporation, USA), an analyser for dissolved ozone (Orbisphere 3600, Orbisphere Laboratories, USA) and computer programs for recording the data (Ozone, Tirkkonen & Löfström, Finland; Moca 3600, Orbisphere Laboratories, USA). The water flow into the contact column was 2.5 l/min. Hydrogen peroxide was fed into the column 2.5 mmol/h and pure ozone 25 mmol/h to achieve a theoretical molar ratio of 1:10 which was found effective in the pre-trial (data not shown). In the treated water, theoretical ozone concentration was 8 mg/l and the contact time was 6.8 min. Theoretical hydrogen peroxide concentration was kept below 1 mg/l in the treated water which was found safe for eggs in the pre-trial

After the contact column water was piped to a tank where dissolved ozone was removed by air stripping.

2.3. UV

UV disinfection was conducted with ProMinent Dulcodes 45 W low-pressure mercury vapour lamp (Heidelberg, Germany) with the UV dose of 400 mWs/cm^2.

2.4. UV + H_2O_2

UV irradiation was conducted with Advantage 5/5 Plus 40 W low-pressure mercury vapour lamp (Aqua Ultraviolet, Temecula, California, USA). UV dose was 400 mWs/cm^2. Hydrogen peroxide

was fed in concentration of 5 mg/l (0.147 mmol/l) into the water before UV reactor.

2.5. Determination of water quality

The quality of inlet water was monitored from samples taken before the incubation tanks. Four sampling points were analysed: (1) untreated water (which included two treatment groups: control and formalin baths), (2) $O_3 + H_2O_2$, (3) UV, (4) UV + H_2O_2. pH was measured with WTW pH340 meter (Weilheim, Germany) using SenTix 41 electrode. Conductivity was measured with WTW LF330 meter using TetraCon 325 electrode. Dissolved oxygen and temperature were measured with WMT OxyGuard Handy Delta meter (Baton Rouge, Louisiana, USA). $UV_{253.7}$-absorbance was measured with Shimadzu UV-mini 1240 spectrophotometer (Kyoto, Japan). Chemical oxygen demand (COD_{Mn}) was analysed according to the Finnish Standards SFS 3036 (SFS 3036). Hydrogen peroxide was analysed using the spectrophotometric method of Tanner and Wong (Tanner and Wong, 1998). Organic acids (acetate, formate) were analysed by an ion chromatographic method of Rantakokko et al. (2004).

2.6. Microbial analysis

Fifteen eggs were taken from each tray with sterile spoon and they were transferred to tubes containing 9 ml of sterile peptone saline water (0.1% peptone, 0.9% NaCl). Tubes were vortexed for 1 min and samples were serially diluted into 10^{-8}, 100 μl of the solution was spread in duplicate into R2A-agar plates (LAB M, Bury, England). Plates were incubated in +15 °C for 7 days. Microbial analyses were done at 4 time points (day 0, day 1, day 10 and day 20), until eggs reached eyed-egg stage.

2.7. Microbial diversity analysis

Microbial community structure on the fish eggs in each treatment was studied using universal length heterogeneity analysis of PCR amplified 16S rRNA genes (LH-PCR, Suzuki et al., 1998). Egg samples were collected from each tank into 1.5 ml sterile tubes and stored frozen (−20 °C) until analysis. In the laboratory, the tubes were taken to room temperature and five eggs from each tube were subjected to DNA extraction with Biosprint 15 DNA Blood kit (QIAGEN) and a KingFisher magnetic particle separator (Labsytems). Bacterial 16S rRNA genes were amplified using broad range bacterial PCR primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Weisburg et al., 1991) labeled with IRD-700 label and Prun518 (5′-ATT ACC GCG GCT GCT GG -3′) (Muyzer et al., 1993). The PCR reaction mixture contained 0.3 mM dNTPs, 0.3 μM of each primer, 1× Biotools PCR buffer, 1 U Biotools DNA-polymerase and 1 μl of DNA extracted from the eggs. The PCR included an initial denaturation step at 95 °C for 5 min, 30 cycles of amplification (94 °C for 30 s, 55 °C for 60 s, 72 °C for 180 s), and a final extension step at 72 °C for 15 min. These LH-PCR products were run in a 6% Long Ranger denaturating polyacrylamide gel (FMC Bioproducts, Rockland, ME) with a LI-COR 4200 automatic sequencer (LI-COR BioTech, Lincoln, NE). Quantity One software (Bio-Rad Laboratories, Hercules, CA) was used for the analysis of the bacterial diversity data. Each LH-PCR band generated in LH-PCR and representing more than 0.5% of the total area in the fragment analysis was considered as an operational taxonomical unit (OTU) with a specific length rounded to the closest integer.

2.8. Statistical analyses

One-way ANOVA test was used to test differences between the treatments and two-sided Dunnett's *t*-test to test differences between treatment groups against control group. Differences in the OTU profiles between treatments were analyzed using ANOVA with LSD post hoc test and hierarchical cluster analysis, which was performed to calculate the similarity and squared Euclidian distance for each sample. All statistical analyses were performed with SPSS for Windows, (versions 13.0 and 14.01).

3. Results

3.1. Incubation result

Ultraviolet irradiation of inlet water significantly decreased mortality ($p = 0.011$) during the 28 days incubation. Highest mortalities were found in the control group (77.3%) while lowest were found from treatment 4 (UV) (14.3%) (Fig. 5). The other treatments and formalin baths decreased rainbow trout egg mortality compared to the control, but the results were not statistically significant. *Saprolegnia* infected eggs were found in every tray during the trial. First infected eggs were visually detected after 7 days and after 10 days at least one infected egg was detected in every tray. However, cumulative appearance of infected eggs (Fig. 6) shows that UV irradiation of inlet hatchery water delayed appearance of *Saprolegnia* infection, but this result was not statistically significant.

3.2. Water quality

The quality of the inlet water in all treatment groups was uniform during the whole trial in respect of pH, conductivity,

Table 1
Physical and chemical characteristics of the inlet water after different treatments. The values of the parameters that represent organic matter (COD_{Mn}, UV-absorbance, acetate, formate) are presented from the first and the last day of the trial. A value of untreated water comprises water for control and formalin treated hatching trays (treatments 1 and 2). COD_{Mn} could not be analyzed from treatments 3 ($O_3 + H_2O_2$) and 5 (UV + H_2O_2) because H_2O_2 interferes the analysis.

Parameter average ±SD (*n*)	Untreated water	$O_3 + H_2O_2$	UV	UV + H_2O_2
pH	6.9 ± 0.1 (12)	6.7 ± 0.1 (12)	6.9 ± 0.1 (12)	6.9 ± 0.1 (12)
Conductivity [μS/cm]	57 ± 1 (12)	59 ± 1 (11)	57 ± 1 (12)	57 ± 1 (12)
Temperature [°C]	10 ± 0.3 (12)	10 ± 0.5 (12)	10 ± 0.4 (12)	10 ± 0.6 (12)
Oxygen content [mg/l]	11 ± 0.4 (4)	27 ± 2.7 (4)	10 ± 0.2 (4)	11 ± 0.6 (4)
H_2O_2 concentration [mg/l]	na.	0.7 ± 0.1 (12)	na.	4.4 ± 0.2 (11)
COD_{Mn} (A) [mg/l]	12.7 (1)	na.	11.8 (1)	na.
COD_{Mn} (B)[mg/l]	16.4 (1)	na.	15.8 (1)	na.
UV-absorbance (A) [1/cm]	0.44 (1)	0.26 (1)	0.43 (1)	0.43 (1)
UV-absorbance (B) [1/cm]	0.55 (1)	0.33 (1)	0.53 (1)	0.53 (1)
Acetate (A) [μg/l]	26 (1)	240 (1)	32 (1)	57 (1)
Acetate (B) [μg/l]	122 (1)	372 (1)	133 (1)	159 (1)
Formate (A) [μg/l]	36 (1)	366 (1)	39 (1)	111 (1)
Formate (B) [μg/l]	52 (1)	459 (1)	70 (1)	304 (1)

Symbols: COD_{Mn} = chemical oxygen demand, na. = not analysed, (A) = measured at the beginning of the trial, (B) = measured at the end of the trial.

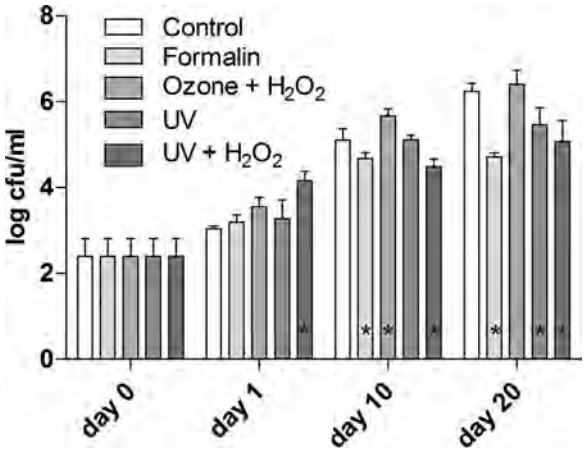


Fig. 2. Total aerobic bacterial counts of surface of rainbow trout eggs during the incubation trial (mean + SD, $n = 3$). Significant differences compared to control (ANOVA, Dunnet's post hoc test) are presented with *($p < 0.05$).

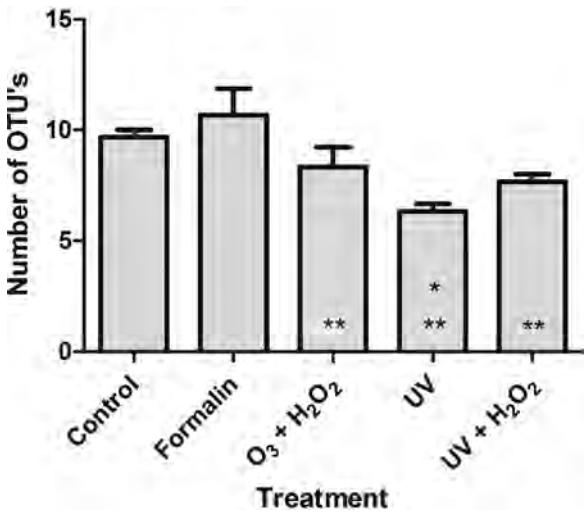


Fig. 3. Average number (mean ± S.E., $n = 3$) of operational taxonomical units (OTU's) in surface microbiota of fish eggs detected by LH-PCR. Significant differences compared to control (ANOVA, LSD post hoc test) are presented with *($p < 0.05$), while significant differences compared to formalin baths are presented with **($p < 0.05$).

temperature, oxygen content and hydrogen peroxide concentration (treatment groups $O_3 + H_2O_2$ and UV + H_2O_2) (Table 1). The ozonized water was supersaturated by oxygen (saturation grade about 240%). In Table 1 the values of parameters that represent organic matter (COD_{Mn}, UV-absorbance, acetate, formate) are presented only from the first and the last day of the trial. The increase of the organic matter in the water resulted from the spring turnover of the lake Kallavesi. COD_{Mn} and UV-absorbance increased about 30% during the trial. COD_{Mn} could not be analysed from the treatments 3 and 5 since H_2O_2 interfered the analysis. The concentrations of acetate and formate increased also during the trial. $O_3 + H_2O_2$-treatment decreased the UV-absorbance by 40%, but UV- and UV + H_2O_2-treatments did not have an influence on that parameter. $O_3 + H_2O_2$-treatment produced almost up to ten-fold increase in acetate and formate concentrations. When water was treated with UV + H_2O_2, up to fivefold increase in the formate and 30–120% increase in the acetate concentration occurred. UV-treatment did not have any significant effect on the concentrations of acetate or formate.

3.3. Microbial analysis

Progressive increase in the total aerobic bacterial counts was detected in all the control and treatment groups during the trial (Fig. 2). Slight differences in the bacterial counts could be found even after one day incubation, when highest bacterial levels were found in UV + H_2O_2 group (4.16 log cfu/ml) and lowest in control group (3.03 log cfu/ml). However, after 10 days and 20 days incubation highest bacterial counts were in $O_3 + H_2O_2$ group (5.67 log cfu/ml and 6.41 log cfu/ml, respectively) and lowest in the UV treated units. As expected, formalin treatments also decreased bacterial levels on the egg surface.

3.4. Microbial diversity analysis

The number of OTU's representing the number of dominant microbial groups with the LH-PCR resolution (length variation 465–565 bp, Tiirola et al., 2003) differed significantly between treatments (ANOVA $F = 5.6$, $df = 4$, $p = 0.012$) (Fig. 3). Hierarchical cluster analysis revealed that treatments had a significant effect on the bacterial community structure (Fig. 4). Bacterial diversity in group 5 (UV + H_2O_2) was distinctively different from the other groups and all replicates of group 3 seemed to locate in same branch.

4. Discussion

To prevent *Saprolegnia* sp. infection in rainbow trout egg hatching it is essential to pay attention on the initial infection by destroying fungus spores from the inlet water. In this study three disinfection methods ($O_3 + H_2O_2$, UV, UV + H_2O_2) were therefore tested for the treatment of the inlet water for decreasing the *Saprolegnia* sp. infection. High dose UV-irradiation (400 mWs/cm^2) of inlet water decreased mortality of rainbow trout eggs during the experiment and was capable to delay appearance of *Saprolegnia* infection on the eggs. As far as we know, this study is the very first to report hatching success and *Saprolegnia*-infection of salmonid eggs after inlet water treatment by UV. Water demands at hatcheries are usually relatively low, so costs of high dose of UV-irradiation stay economically reasonable.

Variations in rainbow trout egg mortalities between the replicates during the hatching trial were high, especially in the control group and treatments 3 ($O_3 + H_2O_2$) and 5 (UV + H_2O_2), although the egg quality and fertilization rate were good. High standard

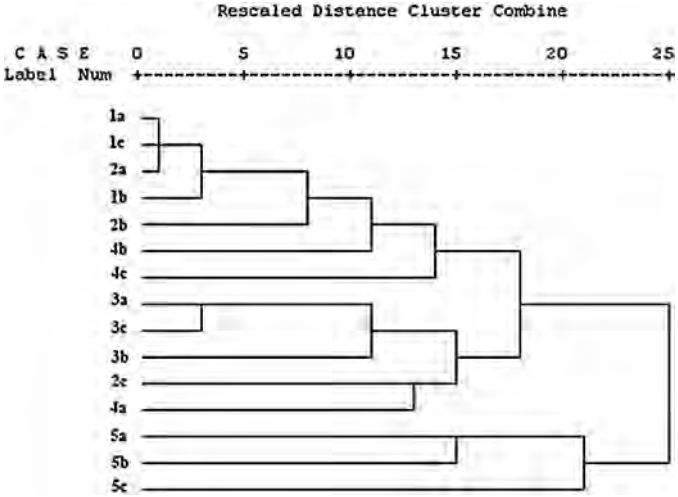


Fig. 4. Hierarchical cluster analysis of the surface microbiota of fish eggs detected by LH-PCR. Treatments 1–5 are 1 = control, 2 = formalin bath, 3 = $O_3 + H_2O_2$, 4 = UV irradiation, 5 = UV irradiation + H_2O_2, a, b and c indicate replicates from each treatments.

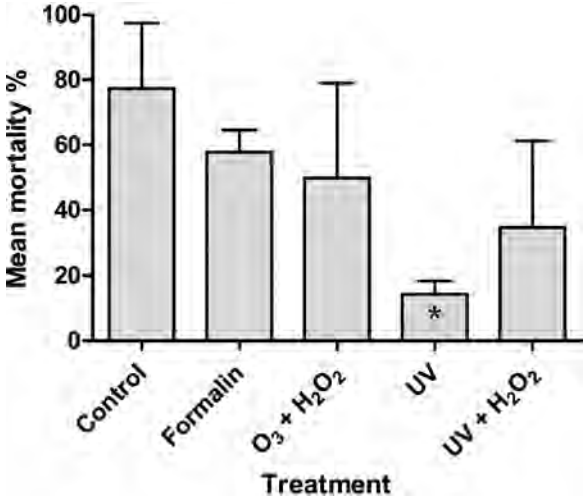


Fig. 5. Mortality percentage of rainbow trout egg incubation trials represented as (mean + SD, $n = 3$). Significant difference compared to control (ANOVA, Dunnet's post hoc test) is presented with *($p < 0.05$).

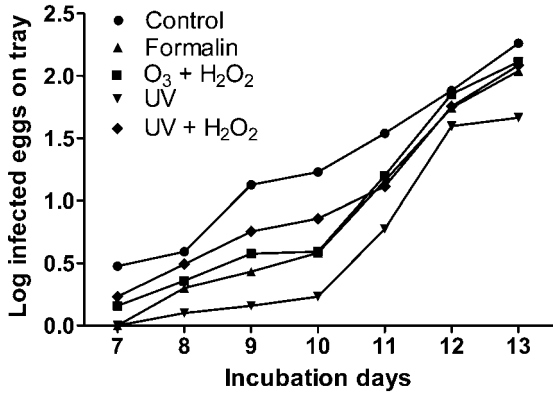


Fig. 6. Logarithmic cumulative appearance of infected eggs on hatching trays in different treatment ($n = 3$) during the incubation days 7–13.

deviations have been detected in several incubation trials with eggs of salmonid species (Waterstrat and Marking, 1995; Arndt et al., 2001; Forneris et al., 2003). Hierarchical cluster analysis revealed that bacterial diversity of treatments 3 and 5 differed from other groups, suggesting that H_2O_2 had remarkably altered bacterial composition of the inlet water affecting the microbial biodiversity on the eggs. Hydrogen peroxide concentration was determined according to the results of pre trials so that only safe levels of H_2O_2 would contact the eggs. Intensive studies on effects of oxygen super saturation on physiology and stress of salmonid species have been made (Ritola et al., 2002a, 2002b), but effects of hyperoxia on fish eggs is still poorly reported. Hence, the effects of increased oxygen content of hatchery water by ozonation and H_2O_2 degradation on egg mortality or egg-associated bacteria are difficult to evaluate.

Sampling during the incubation trial may have slightly increased egg mortality, since taking the egg samples may have caused disturbance to eggs and also affected the *Saprolegnia* spreading inside the tray. However, the procedure was identical for all trays.

In this study the formalin treatments were started after the first signs of fungal infestation. No prophylactic formalin treatment was applied, because in 2005 (the time of the experiment) this practice was not encouraged by National Veterinary and Food Research Institute (EELA, from May 2006 onwards Finnish Food Safety Authority EVIRA) and the FGFRI (Finnish Game and Fisheries Research Institute) complied with this recommendation.

The applied UV dose (400 mWs/cm^2) in the trial was very high compared to the doses usually employed in drinking water disinfection (around 30–40 mWs/cm^2). The high dose was necessary for two reasons: (1) the transmission efficiency of lake water is much lower than that of drinking water which decreases the absorption of UV irradiation into the micro-organisms (Wolfe, 1990), and (2) *Saprolegnia* is capable of producing encysted zoospores, which are normally more resistant to ultraviolet irradiation than the vegetative cells of bacteria (Wedemeyer, 1996). It was also necessary to use high doses of ozone (8 mg/l), because the high organic matter content in the lake water causes high ozone depletion (Von Gunten, 2003a). UV irradiation and ozone treatments were combined with the addition of low concentrations of H_2O_2 to attain synergistic disinfection efficiency. In pre-trials, 5 mg/l H_2O_2 concentration was observed to be safe for rainbow trout eggs and therefore this was the maximum concentration used in this study.

Based on the reduction of cultured heterotrophic bacteria on the fish eggs the best disinfection efficiency was attained with UV + H_2O_2, while O_3 + H_2O_2 was the least effective treatment. Indeed, O_3 + H_2O_2 slightly increased heterotrophic bacteria levels on egg surfaces compared to the controls. Ozonation causes organic matter degradation (Volk et al., 1997), which could be observed as a decrease in UV-absorbance of the treated water in this trial. As a result of this degradation, smaller molecular weight organic compounds, such as aldehydes, ketones and organic acids, are formed (Von Gunten, 2003b). In this study, the concentrations of the small organic acids acetate and formate were measured and they increased in the water treated with O_3 + H_2O_2. Hammes et al. (2006) found similar increases in acetate and formate levels in ozonated lake water, and that the lake water spiked with phytoplankton increases the organic acid concentrations after ozonation due to leakage of the cells and further oxidation of cytoplasmic organic compounds (Hammes et al., 2007). Similarly, also in this study, the increase of organic matter (indicated by the increase of COD_{Mn}) at the end of the spring turnover coincided with increased levels of short chain organic acids in O_3 + H_2O_2 treated water. Some increase of both organic acids was also observed as a result of UV + H_2O_2 treatment, but not when water was solely UV irradiated. Therefore even the low level (5 mg/l) of hydrogen peroxide can cause degradation of the organic matter in the water. Small molecular weight organic compounds are considered more biodegradable than bigger ones (Schmidt et al., 1998; Hu et al., 1999), and they may therefore serve as substrates for the heterotrophic bacteria. By increasing the ozone dose, better disinfection efficiency could be attained, but decomposition of soluble ozone in the water would become more difficult. UV-treatment had a somewhat lower disinfection efficiency than UV + H_2O_2, which might be due to the lack of hydrogen peroxide (Wagner et al., 2008, 2012).

Bacterial counts increased progressively during the incubation period, which has also been detected earlier by Barker et al. (1989) with rainbow trout (*O. mykiss*) and brown trout (*Salmo trutta*) and by Barnes et al. (1999) with inland fall Chinook salmon (*Oncorhynchus tshawytscha*). Appearance of dead eggs on incubation trays increase amounts of available nutrients in water which may partly explain progressive increase in the bacterial counts.

Bacterial levels were decreased by formalin baths in treatment 2. However, it is interesting to note that number of OTU's increased in treatment compared to control. Formalin may selectively cut down some of the dominant bacteria on surface of the eggs and hence new bacterial species have possibility to colonize the egg surface. Wagner et al. (2008) found that single bath treatment with 1000 mg formalin/l was not sufficient to decrease bacterial levels on rainbow trout egg surfaces with. In this trial, eggs were formalin bathed (1000 mg/l, 15 min) altogether 8 times prior to sampling on day 20, which may partly explain the difference.

Hierarchical cluster analysis showed that egg-associated microbes in treatment 5 ($UV + H_2O_2$) significantly differed from other groups and all the replicates of group 3 ($O_3 + H_2O_2$) were located in same branch, which suggests the role of hydrogen peroxide as modifier of inlet water, likely favouring catalase positive bacteria (Nakayama et al., 2008).

As a conclusion, UV irradiation of inlet water with high dose ($400\,mWs/cm^2$) seems to provide potential method to decrease *Saprolegnia* infection on rainbow trout eggs and bacterial load on eggs. *Saprolegnia* sp. is able to colonize surface of the dead eggs and to suffocate the surrounding living eggs (Kitancharoen and Hatai, 1996). Rainbow trout eggs are sensible to movement during the pre-eyed egg stage. Hence removal of the dead eggs is possible only when incubating eggs in vertical tray incubator, but not in multilayer incubation systems, which are normal procedures in majority of rainbow trout hatcheries. Every additional day postponing *Saprolegnia* hyphae appearance in incubation tray increases the hatching result remarkably, while dead eggs, which provide nutrients and growth surface for *Saprolegnia*, can be removed after eyed egg stage. Treatment of inlet water modifies microbial community structure on surface of rainbow trout eggs and may promote conditions to favour growth of certain bacterial strains on the egg surface. Several bacterial strains, especially from genus *Aeromonas* (Lategan and Gibson, 2003; Lategan et al., 2004, 2006) and *Pseudomonas* (Bly et al., 1997) produce substances, which prevent or delay *Saprolegnia* growth. Future studies should be aimed to find such protective bacterial cultures to be used together with UV irradiation.

Acknowledgements

The authors want to acknowledge the skilful assistance of laboratory and technician personnel in Department of Biosciences and Finnish Game and Fisheries Research Institute and Dr. Marja Tiirola from University of Jyväskylä for giving insightful comments. Financial support for these experiments was provided by Kemira Grow-How Oy, Savon Taimen Oy and Ministry of Agriculture and Forestry, Finland.

References

Arndt, R.E., Wagner, E.J., Routledge, M.D., 2001. Reducing or withholding hydrogen peroxide treatment during a critical stage of rainbow trout development: Effects on eyed-eggs, hatch, deformities and fungal control. North American Journal of Aquaculture 70, 118–127.

Barker, G.A., Smith, S.N., Bromage, N.R., 1989. The bacterial flora of rainbow trout, *Salmo gairdneri* Richardson, and brown trout *Salmo trutta* L., eggs and its relationship to developmental success. Journal of Fish Diseases 12, 281–293.

Barker, G.A., Smith, S.N., Bromage, N.R., 1990. Effect of oxolinic acid on bacterial flora and hatching success rate of rainbow trout, *Oncorhynchus mykiss*, eggs. Aquaculture 91, 205–222.

Barker, G.A., Smith, S.N., Bromage, N.R., 1991. Commensal bacteria and their possible relationship to the mortality of incubating salmonid eggs. Journal of Fish Diseases 14, 199–210.

Barnes, M.E., Gabel, A.C., Cordes, R.J., 1999. Bacterial populations during inland fall chinook salmon egg culture in vertical-flow tray incubators. North American Journal of Aquaculture 61, 252–257.

Barnes, M.E., Wintersteen, K., Sayler, W.A., Cordes, R.J., 2000. Use of formalin during incubation of rainbow trout eggs. North American Journal of Aquaculture 62, 54–59.

Barnes, M.E., Bergmann, D., Stephenson, H., Gabel, M., Cordes, R.J., 2005. Bacterial numbers from landlocked fall chinook salmon eyed eggs subjected to various formalin treatments as determined by scanning electron microscopy and bacteriological culture methods. North American Journal of Aquaculture 67, 23–33.

Bhalla, D.K., 1999. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. Journal of Toxicology and Environmental Health Part B: Critical Reviews 2, 31–86.

Blomberg, A., Mudway, I., Nordenhall, C., Hedenstrom, H., Kelly, F., Frew, A., Holgate, S., Sandstrom, T., 1999. Ozone-induced lung function decrements do not correlate with early airway inflammatory or antioxidant responses. The European Respiratory Journal 13, 1418–1428.

Bly, J.E., Quiniou, S., Lawson, L.A., Clem, L.W., 1997. Inhibition of *Saprolegnia* pathogenic for fish by *Pseudomonas fluorescens*. Journal of Fish Diseases 20, 35–40.

Brown, C., Russo, D.J., 1979. Ultraviolet light disinfection of shellfish hatchery sea water: I. Elimination of five pathogenic bacteria. Aquaculture 17, 17–23.

Caruana, S., Yoon, G.H., Freeman, M.A., Mackie, J.A., Shinn, A.P., 2012. The efficacy of selected plant extracts and bioflavonoids in controlling infections of Saprolegnia australis (Saprolegniales; Oomycetes). Aquaculture 358/359, 146–154.

Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011. The effects of ozone and water exchange rates on water quality and rainbow trout *Oncorhynchus mykiss* performance in replicated water recirculating systems. Aquacultural Engineering 44, 80–96.

Emerick, R.W., Loge, F.J., Thompson, D., Darby, J.L., 1999. Factors influencing ultraviolet disinfection performance. Part II: association of coliform bacteria with wastewater particles. Water Environment Research 71, 1178–1187.

Forneris, G., Bellardi, S., Palmegiano, G.B., Saroglia, M., Sicuro, B., Gasco, L., Zoccarato, I., 2003. The use of ozone in trout hatchery to reduce saprolegniasis incidence. Aquaculture 221, 157–166.

Fregeneda-Grandes, J.M., Rodriguez-Cadenas, F., Aller-Gancedo, J.M., 2007. Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by saprolegniosis. Journal of Fish Biology 71, 510–518.

Gieseker, C.M., Serfling, S.G., Reimschuessel, R., 2006. Formalin treatment to reduce mortality associated with Saprolegnia parasitica in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 253, 120–129.

Good, C., Davidson, J., Welsh, C., Snekvik, K., Summerfelt, S., 2011. The effects of ozonation on performance, health and welfare of rainbow trout *Oncorhynchus mykiss* in low-exchange water recirculation aquaculture systems. Aquacultural Engineering 44, 97–102.

Hammes, F., Salhi, E., Köster, O., Kaiser, H-P., Egli, T., von Gunten, U., 2006. Mechanistic and kinetic evaluation of organic disinfection by-product and assmilable organic carbon (AOC) formation during the ozonation of drinking water. Water Research 40, 2275–2286.

Hammes, F., Meylan, S., Salhi, E., Köster, O., Egli, T., von Gunten, U., 2007. Formation of assimilaböe organic carbon (AOC) and specific natural organic matter (NOM) fractions during ozonation of phytoplankton. Water Research 41, 1447–1454.

Hedrick, R.P., McDowell, T.S., Marty, G.D., Mukkatira, K., Antonio, D.B., Andree, K.B., Bukhari, Z., Clancy, T., 2000. Ultraviolet irradiation inactivates the waterborne infective stages of *Myxobolus cerebralis*: a treatment for hatchery water supplies. Diseases of Aquatic Organisms 42, 53–59.

Hu, J.Y., Wang, Z.S., Ng, W.J., Ong, S.L., 1999. The effect of water treatment processes on the biological stability of potable water. Water Research 33, 2587–2592.

Hussein, M.M.A., Hatai, K., Nomura, T., 2001. Saprolegniosis in salmonids and their eggs in Japan. Journal of Wildlife Diseases 37, 204–207.

Kitancharoen, N., Hatai, K., 1996. Experimental infection of *Saprolegnia* spp. in rainbow trout eggs. Fish Pathology 31, 49–50.

Klestadt, D., Laval-Gilly, P., Falla, J., 2002. Ozone-mediated cytotoxicity after short-term exposure and its relation to the production of cellular metabolites (NO, H_2O_2). Cell Biology and Toxicology 18, 259–269.

Lategan, M.J., Gibson, L.F., 2003. Antagonistic activity of *Aeromonas media* strain A199 against *Saprolegnia* sp., an opportunistic pathogen of the eel, *Anguila australis* Richardson. Journal of Fish Diseases 26, 147–153.

Lategan, M.J., Torpy, F.R., Gibson, L.F., 2004. Control of saprolegniosis in the eel *Anguilla australis* Richardson, by *Aeromonas media* strain A199. Aquaculture 240, 19–27.

Lategan, M.J., Booth, W., Shimmon, R., Gibson, L.F., 2006. An inhibitory substance produced by *Aeromonas media* A199, an aquatic probiotic. Aquaculture 254, 115–124.

Lee, S., Radtke, T., 1998. Exposure to formaldehyde among fish hatchery workers. Applied Occupational and Environmental Hygiene 13, 3–6.

Liltved, H., Hektoen, H., Efraimsen, H., 1995. Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. Aquacultural Engineering 14, 107–122.

Marking, L.L., Rach, J.J., Schreier, T.M., 1994. Evaluation of antifungal agents for fish culture. Progressive Fish-Culturist 56, 225–231.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Nakayama, M., Nakajima-Kambe, T., Katayama, H., Higuchi, K., Kawasaki, Y., Fuji, R., 2008. High catalase production by *Rhizobacterium radiobacter* strain 2-1. Journal of Bioscience and Bioengineering 106, 554–558.

Pottinger, T.G., Day, J.G., 1999. A *Saprolegnia parasitica* challenge system for rainbow trout: assessment of Pyceze as an anti-fungal agent for both fish and ova. Diseases of Aquatic Organisms 36, 129–141.

Rach, J.J., Gaikowski, M.P., Howe, G.E., Schreier, T.M., 1998. Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm- and coolwater fishes. Aquaculture 165, 11–25.

Rach, J.J., Redman, S., Bast, D., Gaikowski, M.P., 2005. Efficacy of hydrogen peroxide versus formalin treatments to control mortality associated with lake trout eggs. North American Journal of Aquaculture 67, 148–154.

Rantakokko, P., Mustonen, S., Yritys, M., Vartiainen, T., 2004. Ion chromatographic method for the determination of selected inorganic anions and organic acids from raw and drinking waters using suppressor current switching to reduce the background noise. Journal of Liquid Chromatography and Related Technologies 27, 829–842.

Ritola, O., Livingstone, D.R., Peters, L.D., Lindström-Seppä, P., 2002a. Antioxidant processes are affected in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ozone and oxygen-supersaturated water. Aquaculture 210, 1–19.

Ritola, O., Tossavainen, K., Kiuru, T., Lindström-Seppä, P., Mölsä, H., 2002b. Effects of continuous and episodic hyperoxia on stress and hepatic glutathione levels in one-summer-old rainbow trout (*Oncorhynchus mykiss*). Journal of Applied Ichthyology 18, 159–164.
Rosenfeldt, E., Linden, K., Canonica, S., vonGunten, U., 2006. Comparison of the efficiency of OH radical formation during ozonation and the advanced oxidation processes O_3/H_2O_2 and UV/H_2O_2. Water Research 40, 3695–3704.
Scannell, C., Chen, L., Aris, R., Tager, I., Christian, D., Ferrando, R., Welch, B., Kelly, T., Balmes, J., 1996. Greater ozone-induced inflammatory responses in subjects with asthma. American Journal of Respiratory and Critical Care Medicine 154, 24–29.
Schmidt, W., Hambsch, B., Petzoldt, H., 1998. Formation of Bioavailable Disinfection By-products. International Water Services Association, Mülheim an der Ruhr, Germany, pp. XVI-1–XVI-12.
Schreier, T.M., Rach, J.J., Howe, G.E., 1996. Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout eggs. Aquaculture 140, 323–331.
Sharrer, M.J., Summerfelt, S.T., Bullock, G.L., Gleason, L.E., Taeuber, J., 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. Aquacultural Engineering 33, 135–149.
Straus, D.L., Mitchell, A.J., Carter, R.R., Steeby, J.A., 2012. Hatch rate of channel catfish *Ictalurus punctatus* (Rafinesque 1818) eggs treated with 100 mg L^{-1} copper sulphate pentahydrate. Aquaculture Research 43, 14–18.
Summerfelt, S.T., Hochheimer, J.N., 1997. Review of ozone processes and applications as an oxidizing agent in aquaculture. Progressive Fish-Culturist 59, 94–105.
Summerfelt, S.T., 2003. Ozonation and UV irradiation – an introduction and examples of current applications. Aquacultural Engineering 28, 21–36.
Suzuki, M., Rappe, M.S., Giovannoni, S.J., 1998. Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Applied and Environmental Microbiology 64, 4522–4529.
Tanner, P.A., Wong, A.Y.S., 1998. Spectrophotometric determination of hydrogen peroxide in rainwater. Analytica Chimica Acta 370, 279–287.
Thoen, E., Evensen, Ø., Skaar, I., 2011. Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. Journal of Fish Diseases 34, 601–608.
Tiirola, M.A., Suvilampi, J., Kulomaa, M.S., Rintala, J., 2003. Microbial diversity in a thermophilic aerobic biofilm process: analysis by length heterogeneity PCR (LH-PCR). Water Research 37, 2259–2268.
Toor, R., Mohseni, M., 2007. UV-H_2O_2 based AOP and its integration with biological activated carbon treatment for DBP reduction in drinking water. Chemosphere 66, 2087–2095.
van West, P., 2006. Saprolegnia parasitica, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20, 99–104.
Volk, C., Roche, P., Joret, J-C., Paillard, H., 1997. Comparison of the effect of ozone, ozone-hydrogen peroxide system and catalytic ozone on the biodegradable organic matter of a fulvic acid solution. Water Research 31, 650–656.
Von Gunten, U., 2003a. Ozonation of drinking water: part I. Oxidation kinetics and product formation. Water Research 37, 1443–1467.
Von Gunten, U., 2003b. Ozonation of drinking water: part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water Research 37, 1469–1487.
Wagner, E.J., Arndt, R.E., Billman, E.J., Forest, A., Cavender, W., 2008. Comparison of the efficacy of iodine, formalin, salt, and hydrogen peroxide for control of external bacteria on rainbow trout eggs. North American Journal of Aquaculture 70, 118–127.
Wagner, E.J., Oplinger, R.W., Bartley, M., 2012. Laboratory and production scale disinfection of salmonid eggs with hydrogen peroxide. North American Journal of Aquaculture 74, 92–99.
Waterstrat, P.R., Marking, L.L., 1995. Clinical evaluation of formalin, hydrogen peroxide, and sodium chloride for the treatment of *Saprolegnia parasitica* on fall chinook salmon eggs. Progressive Fish-Culturist 57, 287–291.
Wedemeyer, G.A., 1996. Physiology of Fish in Intensive Culture Systems. Chapman & Hall, New York.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. Journal of Bacteriology 173, 697–703.
Wolfe, R.L., 1990. Ultraviolet disinfection of potable water. Environmental Science and Technology 24, 768–773.
Wooster, G.A., Martinez, C.M., Bowser, P.R., O'Hara, D.S., 2005. Human health risks associated with formalin treatments used in aquaculture: initial study. North American Journal of Aquaculture 67, 111–113.

Jouni Heikkinen (M.Sc.) is a microbiologist studying effects of potential microbial applications in fish disease prevention. His recent studies are focused on prevention of Saprolegnia infections in salmonid fish and fish eggs.

Satu Mustonen (M.Sc.) is specialized on drinking water disinfection methods and water chemistry.

Päivi Eskelinen (M.Sc.) has been a research scientist in Finnish Game and Fisheries Research Institute until 2008. She studied egg production and hatchery operation methods, specifically egg quality and health promotion questions. Nowadays she works as program leader in "Natural resources and society" program in Finnish Game and Fisheries Research Institute.

Lotta-Riina Sundberg (PhD, Adjunct professor) is a microbiologist studying evolutionary ecological aspects in the evolution of pathogenicity using a fish pathogen Flavobacterium columnare as a model species. She works as a Principal Investigator in Centre of Excellence in Biological Interactions, University of Jyväskylä, Finland. Recently her studies have focused also on genome evolution, social behaviors of bacteria and the effect of antagonistic trophic interactions on bacterial virulence.

Atte von Wright (PhD, University of Sussex UK, 1981) has been since 1998 professor of food biotechnology in the University of Eastern Finland (previously University of Kuopio). His main research interests include lactic acid bacteria, probiotics, food hygiene and food toxiciology.

Journal of Aquatic Animal Health 26:33–42, 2014

ISSN: 0899-7659 print / 1548-8667 online
DOI: 10.1080/08997659.2013.864720

EXHIBIT F-8

ARTICLE

Infectious Salmon Anemia (ISA) Virus: Infectivity in Seawater under Different Physical Conditions

Siri Vike*
Cermaq, Dronning Eufemias gate 16, Oslo N-0102, Norway

Karin Oelckers
EWOS Innovation Chile, Camino a Pargua km 57, Colaco km 5, Calbuco, Chile

Henrik Duesund
Cermaq, Dronning Eufemias gate 16, Oslo N-0102, Norway

Svein Rune Erga
Department of Physics and Technology, University of Bergen, Allegt. 55, Bergen N-5007, Norway

Javier Gonzalez
EWOS Innovation Chile, Camino a Pargua km 57, Colaco km 5, Calbuco, Chile

Børge Hamre and Øyvind Frette
Department of Physics and Technology, University of Bergen, Allegt. 55, N-5007 Bergen, Norway

Are Nylund
Department of Biology, University of Bergen, Thormohlensgt 55, Bergen N-5020, Norway

Abstract

Infectious salmon anemia (ISA) virus (genus *Isavirus*, family Orthomyxoviridae), present in all major salmon producing countries, is the causative agent for a serious and commercially important disease affecting Atlantic Salmon *Salmo salar*. Nearly all ISA outbreaks occur in the marine production phase and knowledge about survival time for ISA virions in seawater is crucial for an adequate strategy to combat the disease. To acquire knowledge about this important factor, a study of ISA virus exposed to four different physical conditions was carried out. The virions' survival was tested in sterile seawater, sterile seawater with normal ultraviolet light radiation (UVR), natural seawater, and natural seawater with UVR. During the 72-h experiment both presence of ISA virus RNA and the infectivity of ISA virions were monitored. The result of this study showed that the infectivity of ISA virions is lost within 3 h of exposure to natural seawater or sterile seawater with UVR. However, it was possible to detect ISA virus RNA throughout the experimental period. This indicates that the effect of both UVR and biological activity of natural seawater limits the survival time of ISA virions under normal conditions. The survival time of ISA virions in sterile seawater was less than 24 h. Based on the available literature and the present study it is not very likely that passive horizontal transmission in seawater over long distances can occur. This is due to the following factors: (1) the effect of UVR and biological activity on ISA virions infectivity found in the present study, (2) the speed and dilution effect in seawater currents in salmon farming areas, (3) the temperature during the major outbreak periods, and (4) the need for an infective dose of ISA virions to reach naive Atlantic Salmon.

*Corresponding author: siri.vike@analytiq.no
Received August 22, 2013; accepted November 7, 2013

Infectious salmon anemia (ISA) virus is an enveloped, single-stranded, negative-sense RNA virus belonging to the genus *Isavirus* (family Orthomyxoviridae) (Koren and Nylund 1997; Mjaaland et al. 1997; Krossoy et al. 1999; King et al. 2012). This virus is the causative agent of ISA (Hovland et al. 1994; Dannevig et al. 1995), which is a serious and commercially important disease causing severe anemia in farmed Atlantic Salmon *Salmo salar*. The first official outbreaks of ISA were registered in Norway in the 1980s (Thorud and Djupvik 1988), and since then the disease has been registered in all major Atlantic Salmon-producing countries: Canada (east) (Mullins et al. 1998; Lovely et al. 1999; Ritchie et al. 2001), UK (Scotland) (Rodger et al. 1998; Rowley et al. 1999), USA (Maine) (Bouchard et al. 2001), Denmark (Faroe Islands) (Lyngøy 2002), and Chile (Kibenge et al. 2001; Godoy et al. 2008).

The ISA viruses from North America are genetically distinct from the ISA viruses of European origin with the exception of one genogroup that occurs on both continents (Devold et al. 2001, 2006; Nylund et al. 2003, 2006; Plarre et al. 2012). The ISA virus isolates from different European countries are distinct but closely related to the first ISA virus detected in Norway (Plarre et al. 2012). The Chilean ISA virus isolates detected during a devastating epizootic in 2007–2009 are also all closely related to Norwegian strains (Kibenge et al. 2009; Vike et al. 2009; Cottet et al. 2010; Plarre et al. 2012). Within the Norwegian Atlantic Salmon production there are several distinct clades of ISA viruses, but they do not form geographically distinct groups.

It has been suggested that the introduction of separate generations during the sea-production phase of salmon farming in the early 1990s in Norway was a major factor for the reduction of horizontal transmission leading to a relatively stable, low number of outbreaks of ISA in the following years. The hygienic measurements that were implemented did, however, not prevent all cases of ISA. Studies based on genotyping of the viruses support the hypothesis that the dominant transmission routes for ISA virus within Norway, after this change in management, are most likely due to vertical transmission and movement of embryos or smolts infected with nonvirulent ISA virus (HPR0) (Nylund et al. 2006; Plarre et al. 2012). This hypothesis is also supported by a study using naturally infected ISA virus-positive broodfish in the production of offspring (fry) that were positive for ISA virus, suggesting vertical transmission of the virus (Søfteland 2005). Vertical transmission of ISA virus may also be the best explanation for the introduction of ISA virus to Chile because the causative agent was of a Norwegian genotype (Vike et al. 2009). Horizontal transmission within populations of Atlantic Salmon has been demonstrated in tanks and cages in both seawater and freshwater (Thorud and Djupvik 1988; Nylund et al. 1993, 1994; Jones and Groman 2001; Thorud and Håstein 2003), and despite the existing evidence of vertical transmission, some researchers still maintain that the ISA virus is only transmitted horizontally and that passive transmission via seawater can explain the movement of ISA virus to proximal sites (Vagsholm et al. 1994; Jarp and Karlsen 1997; Gustafson et al. 2005, 2007; McClure et al. 2005a; Scheel et al. 2007; Lyngstad et al. 2008, 2011; Mardones et al. 2009, 2011; Aldrin et al. 2010, 2011; Murray et al. 2010).

The mechanisms for waterborne transmission of ISA virus are not fully understood, but the virus is known to be present in mucus layers, feces, and urine from the host (Nylund et al. 1994; Totland et al. 1996). Shedding of ISA virus from infected Atlantic Salmon may start as early as 7 d after infection (Totland et al. 1996; Gregory et al. 2009). Gregory et al. (2009) also found that the minimum infective dose of ISA virus in seawater is 1.0×10^1 TCID50/mL (i.e., tissue culture infective dose producing a cytopathic effect in 50% of cultures). This is a relatively low level of infective virions, and such an amount could theoretically reach neighboring farms from a farm experiencing an outbreak of ISA. However, using a very sensitive method that can detect as little as 5.5 ISA virus/mL, Lovdal and Enger (2002) did not detect any ISA virus RNA 80–100 m downstream from a cage with an outbreak of ISA, but did detect the virus inside the cage. The lack of detection after such a short distance from the outbreak cages could be due to a dilution effect or simply be a result of biological or mechanical activity destroying the ISA virions and their genome.

Knowledge about the survival time for ISA virions in freshwater and seawater is limited to a few studies (Nylund et al. 1994; Rimstad and Mjaaland 2002; Tapia et al. 2013; L. A. MacLeod, R. S. Raynard, A. G. Murray, and A. Gregory, [poster presented at the European Association of Fish Pathologists meeting, 2003]). In a review conducted by Rimstad and Mjaaland (2002), they stated that a "3-$\log_{10}$ reduction" in titer of ISA virus after 4 months in seawater at 4°C may occur. However, this claim was not supported by data. The studies presented by Nylund et al. (1994) and Tapia et al. (2013) are of limited relevance for survival of ISA virions under natural conditions due to the experimental design. This is because Nylund et al. (1994) used blood water and Tapia et al. (2013) used sterile water. A nonpeer-reviewed study claimed that ISA virus can remain infective for 1 week in both natural freshwater and seawater at 15°C and 4°C (MacLeod et al., unpublished). Hence, there are no scientific studies published where the infectivity of ISA virions is monitored during exposure to ultraviolet (UV) light radiation (UVR) simulating natural sunlight in natural seawater, i.e. a simulation of natural conditions. The aim of the present study was to gain knowledge about the influence of UV-A (320–380 nm) and UV-B (280–320 nm) radiation and biological activity (defined as seawater containing bacteria, phytoplankton, and zooplankton) on the infectivity of ISA virions.

METHODS

This study was carried out in two parts. The ISA virus was exposed to four different physical conditions during a period of 72 h: natural seawater (NSW) with and without UV (A + B), and sterile seawater (SSW) with and without UV (A + B). The

choice of UV-A and UV-B, excluding UV-C, was based on the existing knowledge about the natural sunlight at sea level (Paul and Gwynn-Jones 2003). The infectivity of the ISA virions, exposed to these different conditions during the experimental period, was tested in a challenge experiment using Atlantic Salmon.

ISA virus culture.—The ISA virus isolate, CH35/09 (accession number: KC905164), obtained from heart tissue of an Atlantic Salmon originating from a sea site in Los Lagos Region, Chile, was selected as the inoculate. The virus was cultured in Atlantic Salmon kidney cells (ASK-cells) with Eagle's Minimum Essential Medium (EMEM; Sigma) supplemented with HEPES buffer (10 mM), nonessential amino acids (0.1 mM/100.0 μM), L-glutamine (4.0 mM), and gentamicin (50.0 μg/mL) without fetal bovine serum (FBS). The ASK-cells were cultured in 75-cm^2 tissue culture flasks (NUNC) at 15°C according to standard protocols (Devold et al. 2000). Virions collected from the supernatant after the third passage were sterile filtered and used in the experiment.

Survival of infective ISA virions.—The survival experiment was carried out at the Department of Physics and Technology at the University of Bergen. The natural seawater (35‰) was collected in the sea west of the island Sotra (Hordaland, Norway) and had no treatment before being added to the beakers. The transport time from Sotra to the laboratory was 1 h. The sterile seawater was filtered, treated with UV, and autoclaved. A constant water temperature of 10°C was maintained in the beakers using a water-bath system. Air was added to each beaker using Maxima 2 × 280 L/T aquarium pumps and an air stone in each beaker. The aeration of the water provided a weak circulation in the beakers. Twelve autoclaved beakers each containing 4 L of seawater were set up in two parallel plastic containers (60 × 40 × 20 cm), six beakers in each. One container was exposed to UVR and contained three beakers with sterile seawater (SSW+UV) and three beakers with natural seawater (NSW+UV). The other container was kept at ambient room light and contained three beakers with sterile seawater (SSW) and three beakers with natural seawater (NSW). Thus, there were four treatments with three replicates each: (1) beakers 1–3: SSW; (2) beakers 4–6: NSW; (3) beakers 7–9; SSW+UV; (4) beakers 10–12: NSW+UV.

In the beakers with UV treatments, the seawater was exposed to UV-A (320–380 nm) and UV-B (280–320 nm) radiation, provided by an array of six UV-A Philips TL20W/05 and five UV-B Philips TL20W/12 fluorescent light tubes providing a maximum intensity at 365 and 312 nm, respectively. The fluorescent light tubes were 55 cm long and arranged such that the distance between each tube of the same type was 13.5 cm (from center to center). The actual UV irradiances during the experiments were measured by a NILU-UV irradiance meter, which is a multichannel radiometer measuring UV irradiances at five channels with center wavelengths of 302, 312, 320, 340, and 380 nm. The following spectral surface UV irradiances were used in the experiments: 0.12 W·m^{-2}·nm^{-1} at 302 nm, 0.13 W·m^{-2}·nm^{-1} at 312 nm, 0.11 W·m^{-2}·nm^{-1} at 320 nm, 0.052 W·m^{-2}·nm^{-1} at 340 nm, and 0.004 W·m^{-2}·nm^{-1} at 380 nm. The UV-B exposure level is representative for average surface irradiances around local noon during summer in western Norwegian coastal waters (Erga et al. 2005).

The experimental period started with the addition of 4.0 mL ISA virions with an estimated titer of 5.0×10^7/mL (TCID50), giving a final titer of approximately 5.0×10^4/mL seawater in each beaker.

Water sampling and filtration.—Sampling was done at the following times after the addition of ISA virions to the beakers: 0, 3, 6, 9, 12, 18, 24, 48, and 72 h. These sampling points are referred to as: T0, T3, T6, T9, T12, T18, T24, T48, and T72 throughout the manuscript. A total of 108 water samples were collected (12 beakers × 9 sampling points), corresponding to 36 different groups. From each beaker, 250 mL were collected with a glass pipette and kept in sterile cell-culture flasks (NUNC) for immediate processing at a neighboring laboratory.

A water-filtering system, consisting of 1MDS electropositive filters using virus–absorption–elution (VIRADEL), was used to concentrate virions from the water samples as described by Andersen et al. (2010). The 250-mL water samples were each divided in two 125-mL subsamples before filtration. The first subsample was used for two purposes: real-time reverse transcription polymerase chain reaction (RT-PCR) and as a back-up sample. The second subsample was used in testing of infectivity in a fish challenge. The filter that was used in real-time RT-PCR analysis and as backup was placed upside down in 1.4 mL lysis buffer (E.Z.N.A total RNA kit, Omega Bio-Tek) in 50-mm petri dishes. Parafilm was used to seal the dishes before gentle shaking at 150 rpm for 10 min at room temperature. Two 350-μL samples were removed from each petri dish using a pipette following the incubation period in the lysis buffer. To one of these samples 4.0 μL of viral hemorrhagic septicemia (VHS) virus was added as an exogenous control to be used in real-time RT-PCR. This sample was further mixed with an equal amount of 70% ethanol (350 μL), vortexed, and frozen at −80°C prior to RNA extraction and real-time RT-PCR analysis. After removal from the petri dish the other sample with concentrated virus in lysis buffer was mixed with an equal amount of 70% ethanol (350 μL), vortexed, and frozen at −80°C for back-up purpose.

The VHS virus strain was used as an exogenous control in order to quantify ISA virus levels at different time points. The selected VHS virus isolate had genotype 3 and was named FA28.02.08 (GenBank accession number: GU121100). This VHS virus was isolated in 2008 from Rainbow Trout *Oncorhynchus mykiss* in western Norway (Duesund et al. 2010). The virus was cultivated at 14°C in Rainbow Trout gill cells (RTgill-W1) for two passages until a cytopathogenic effect with viral endpoint titer of 1×10^7 virus/mL was reached (Duesund et al. 2010). The viral supernatant was sterile filtered, and aliquots were then filled into nine tubes (one for each sampling point) and frozen at −80°C.

The second subsample was placed upside down in beef extract (Sobsey et al. 1985) in 50-mm petri dishes and sealed with parafilm before gentle shaking at 150 rpm for 10 min at room temperature. Two 350-μL samples were taken from each petri dish and directly frozen at −80°C prior to virus isolation (for fish challenge and cell culture).

RNA extraction and real-time RT-PCR on water samples.—The RNA was extracted from each of the water samples using the E.Z.N.A total RNA isolation kit according to the manufacturer's protocol with the modifications described above.

The VHSV08 real-time RT-PCR assay targeting the VHS virus nucleoprotein (N) (Duesund et al. 2010) and the assay ISAV7 (Plarre et al. 2005) targeting segment 7 of the ISA virus were used to detect the RNA from VHS virus and ISA virus, respectively. VersoTM 1-step QRT-PCR ROX Kit was used for the real-time RT-PCR assays, modified for each reaction as follows: 6.25 μL 5× buffer, 0.625 μL RT-Enhancer, 0.125 μL enzyme mix, 2 μL template, and diethylpyrocarbonate-treated water up to the final volume of 12.5 μL. The primer and probe volumes were adjusted to the optimized concentration for each assay as described below. Nontemplate controls and positive controls were used in all assay runs. Analysis was performed in an ABI 7500 sequence detection system (Applied Biosystems). The reaction run for 15 min at 95°C (polymerase activation step) was followed by 45 cycles of 95°C for 15 s (DNA dissociation) and 60°C for 1 min (annealing and elongation). The threshold was fixed at 0.1 for all runs. The *Ct*-value refers to the cycle number at which the change in fluorescence exceeded the fixed threshold during real-time RT-PCR. All real-time RT-PCR samples were run in triplicates and no cut-off was used in the analysis.

Relative quantification of ISA virus RNA.—Both assays were optimized with regard to concentrations of primers and probes prior to real-time RT-PCR analysis. An efficiency test was performed for the ISAV7 and VHSV08 assays in order to do a relative quantification of the amount of ISA virus RNA in the samples. This test was carried out by using a twofold dilution series for VHS virus and a fivefold dilution series for the ISA virus. The templates for the efficiency test were obtained from cell cultures. The dilution series were analyzed in triplicates. The mean *Ct*-value for each triplicate was calculated and a standard curve was made by plotting *Ct*-values against the serial logarithmic dilutions. The amplification efficiency (E) for the ISAV7 and VHSV08 assays were calculated using the formula: $[\text{dilution factor}^{(-1/-\text{slope})}] - 1$. The regression values (R^2) of the different assays were for ISAV7: 0.9989, and for VHSV08: 0.989. The efficiency (E) of the ISAV7 and VHSV08 assays were $E = 0.9461$ and $E = 0.977226$, respectively.

Relative quantification (normalization) of viral RNA from ISA virus in the water samples was calculated using the Microsoft Excel-based computer software Q-Gene (Muller et al. 2002). The *Ct*-values from the ISAV7 assay were normalized against the *Ct*-values from the VHSV08 assay (exogenous control). The results of mean normalized expression (MNE) values (from triplicate real-time RT-PCR analysis) were transformed into a fold increase by defining the lowest MNE value obtained during the experiment of all the water samples as one. The data were then $\log_2$ transformed. In order to visualize these data in graphs they were imported into the GraphPad Prism 6.02 software for Windows (GraphPad Software).

Challenge experiment: infectivity of the ISA virions.—The challenge experiment was done at the Industrilaboratoriet (ILAB) located in Bergen, Norway. Atlantic Salmon, adapted to full-strength seawater, came from a local smolt producer in Hordaland and had an average weight of approximately 150 g. The fish were acclimatized for 1 week in the laboratory prior to the challenge. Ten fish were screened by real-time RT-PCR for presence of ISA virus and VHS virus prior the challenge and they were all found to be negative for the presence of these pathogens. The challenge experiment had been approved by the Norwegian Animal Research Authorities (NARA; ID2832).

In total, 220 fish were included and distributed into eight tanks at 20–30 fish per tank (see Table 1 for details). The fish were injected intraperitoneally (i.p.) with 0.2 mL of the filtered seawater samples (concentration of virions) collected at different time points from the 12 different beakers. Time points included were: T3, T6, T9, T12, T24, T48, and T72. Time points T0 and T18 were left out due to limited capacity in the challenge facility. The challenge experiment included 28 fish groups that received different inocula. The inocula were prepared from the filtrates dissolved in beef extract with 300 μL of EMEM added resulting in a total amount of 1,000 μL for each sample. Samples of 200 μL from each time point were injected into each of five Atlantic Salmon. Each tank received 10 fish of which five were injected i.p. with 0.2 mL EMEM while the last five were not handled (control). Prior to injection all fish were anesthetized with Metacaine MS-222 (tricaine methanesulfonate). To distinguish between different fish groups in the same tank the fish were marked with alcian blue dye (Panjet) color spots at different places at the abdomen. The five untreated control fish were not marked.

The Atlantic Salmon in the experiment were sampled at two different times: (1) three fish were sampled 7 d after the challenge, and (2) two fish were sampled after 14 d. At the time of sampling the fish was killed with a blow to the head and examined for signs of disease. Tissue samples from the gills, heart, and kidney were collected in duplicate in separate tubes (NUNC) for real-time RT-PCR. For reisolation of the virus, subsamples of the kidney and heart were collected in Falcon tubes. All samples were directly frozen at −80°C.

In parallel with the fish challenge trial was the test of infectivity of the same material (seawater from different time points and treatments) in ASK-cells culture. These tests failed; the beef extract had a toxic effect on the ASK-cells used in culture and thus could not be used.

Real-time RT-PCR testing of fish tissue.—Extraction of RNA from the heart tissues was done using Trizol reagent (Invitrogen) as described by Devold et al. (2000). For real-time RT-PCR, the assay ISAV7 was used to detect ISA virus RNA, targeting

TABLE 1. Tank distribution and numbers of Atlantic Salmon challenged with different inocula. The inocula came from time points T3, T6, T9, T12, T24, T48, and T72 from all the four physical conditions: SSW (sterile seawater), SSW+UV (sterile seawater + UV), NSW (normal seawater), and NSW+UV (normal seawater + UV). A total of 28 different inocula were used. Each tank contained 10 control fish in addition to challenged fish. N = number of fish.

Tank	Inocula	N	Total N	Tank	Inocula	N	Total N
1	SSW T3	5	30	5	SSW+UV T3	5	30
	SSW T6	5			SSW+UV T6	5	
	SSW T9	5			SSW+UV T9	5	
	SSW T12	5			SSW+UV T12	5	
2	SSW T24	5	25	6	SSW+UV T24	5	25
	SSW T48	5			SSW+UV T48	5	
	SSW T72	5			SSW+UV T72	5	
3	NSW T3	5	30	7	NSW+UV T3	5	30
	NSW T6	5			NSW+UV T6	5	
	NSW T9	5			NSW+UV T9	5	
	NSW T12	5			NSW+UV T12	5	
4	NSW T24	5	25	8	NSW+UV T24	5	25
	NSW T48	5			NSW+UV T48	5	
	NSW T72	5			NSW+UV T72	5	

segment 7 (Plarre et al. 2005). The endogenous control was an assay for Atlantic Salmon elongation factor alpha, $EF1A_A$, a housekeeping gene described by Olsvik et al. (2005). Real-time RT-PCR was performed as described for the water samples, except that triplicate analyses were not done.

As a quality control of the analyses, an accredited diagnostic real-time RT-PCR laboratory in Norway, Pharmaq Analytiq (www.pharmaq.no), tested approximately half of the samples in parallel. Pharmaq Analytiq used the same assay specific for ISAV segment 7, as described by Plarre et al. (2005). An endogenous control assay for the Atlantic Salmon $EF1A_A$ was included and performed by the method of Olsvik et al. (2005).

RT-PCR and sequencing.—The RT-PCR was performed using Qiagen One-step RT-PCR kit (Qiagen) according to manufacturer's protocol, with the following modifications in the amplification: 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min. The primers used for RT-PCR and sequencing were published by Devold et al. (2001) and Cunningham et al. (2002). In addition, the following primers were used: HAnsF3: CAT CCCAACTTCGATGACACTGG; S6F1: GCAAAGATGGCA CGATTC; HAnsR3: TCCCAAAACCTGCTACACCC. Sequencing was performed using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). All PCR products were sequenced in both directions. The PCR products were purified with E.Z.N.A. PCR cycle pure (Omega Bio-Tek) and illustra ExoStar 1-Step (VWR). Sequencing was performed at the sequencing facility at the University of Bergen (http://seqlab.uib.no/). Sequence data were analyzed and assembled using VectorNTI software version 9.0.0 (Invitrogen) and deposited in the GenBank.

RESULTS

Detection of ISA Virus RNA in Seawater

Water samples taken from the 12 beakers at all sampling points were positive for the presence of ISA virus RNA throughout the entire 72-h experimental period, based on real-time RT-PCR results. Figures 1–4 show the mean normalized expression values (relative amount) of ISA virus RNA for each of the four physical conditions at all the selected time points. The ISA virus

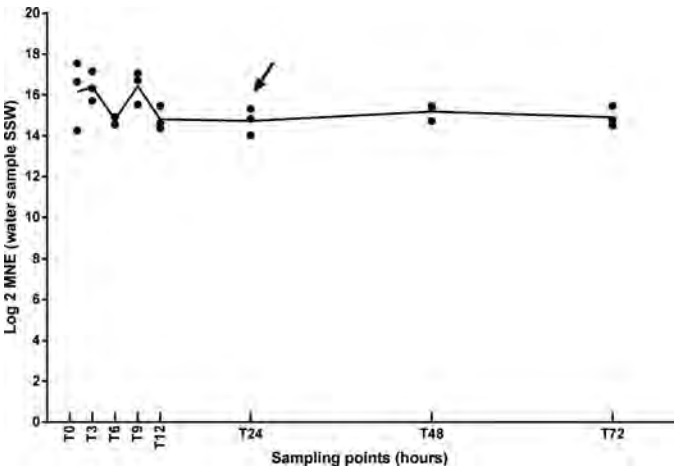


FIGURE 1. Level of ISA virus RNA in sterile seawater (SSW) from all three beakers at all sampling points. Values are normalized against an endogenous control (VHS virus), transformed into a fold increase by a $\log_2$ transformation. The arrow indicates the first sampling point where infectivity was lost.

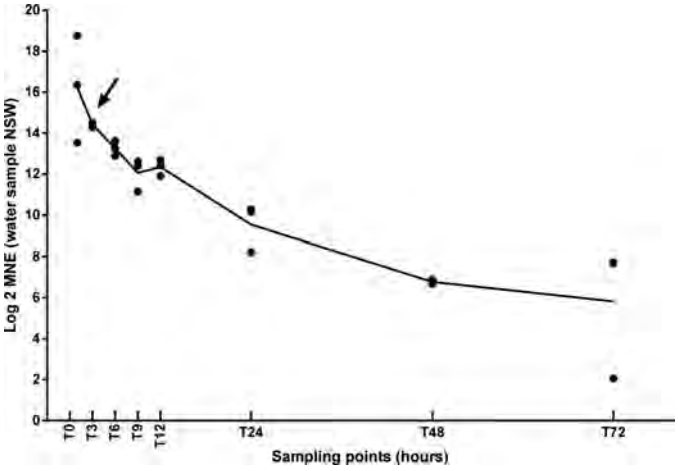


FIGURE 2. Level of ISA virus RNA in normal seawater (NSW) from all three beakers at all sampling points. Values are normalized against an endogenous control (VHS virus), transformed into a fold increase by a $\log_2$ transformation. The arrow indicates the first sampling point where infectivity was lost.

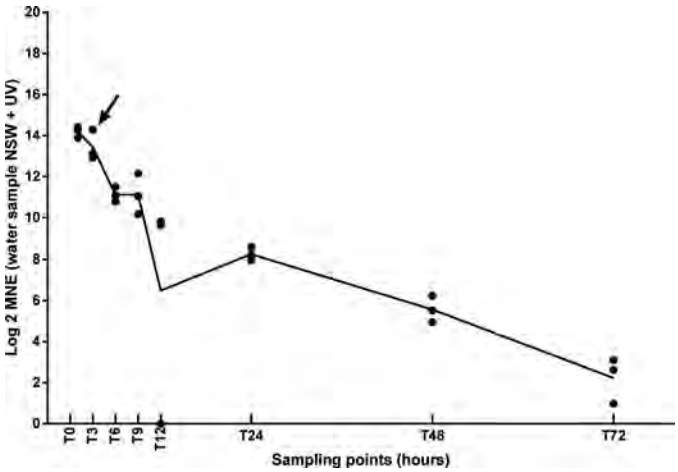


FIGURE 4. Level of ISA virus RNA in natural seawater with UVR (NSW+UV) from all three beakers at all sampling points. Values are normalized against an endogenous control (VHS virus), transformed into a fold increase by a $\log_2$ transformation. The arrow indicates the first sampling point where infectivity was lost.

Ct-values are normalized against the *Ct*-values of the exogenous control (VHS virus), transformed into a fold increase, and are $\log_2$ transformed to better visualize the result. Each time point in the curves is based on mean normalized *Ct*-values that are averages of the triplicates analyzed from the beakers. The points are included to show the variance in the material, and the line shows the mean values.

The beakers containing SSW without UVR had approximately the same level of ISA virus RNA throughout the whole experiment. For the other three physical conditions there was a gradual reduction in the relative amount of ISA virus RNA from start to end (T0 to T72). The relative amount of virus RNA in NSW alone or in SSW exposed to UVR had approximately the same rate of decay throughout the experiment (Figures 2, 3). As expected, the greatest loss in virus RNA occurred in NSW exposed to UVR (Figure 4).

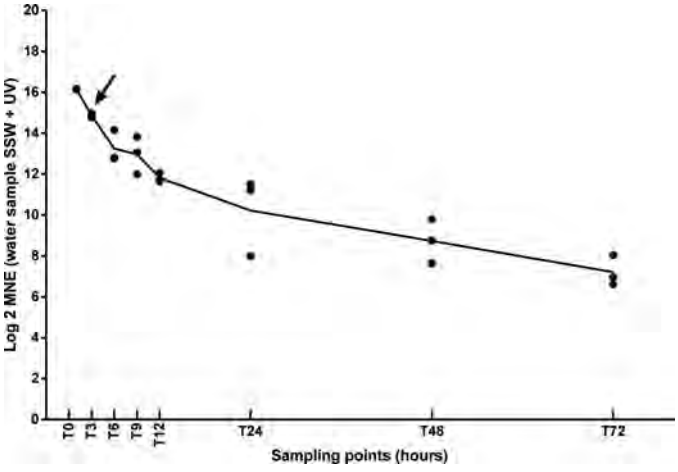


FIGURE 3. Level of ISA virus RNA in sterile seawater with UVR (SSW+UV) from all three beakers at all sampling points. Values are normalized against an endogenous control (VHS virus), transformed into a fold increase by a $\log_2$ transformation. The arrow indicates the first sampling point where infectivity was lost.

Test of ISA Virions Infectivity

Atlantic Salmon challenged with SSW filtrates were infected with ISA virus, while none of the other groups (SSW+UV, NSW, NSW+UV) tested positive for the virus (Table 2). The ISA virions in the SSW group maintained infectivity for at least 12 h (T12), while the T24 sample, collected 24 h after the start of the experiment, did not contain virions able to establish an infection in Atlantic Salmon. A total of 14 fish from the SSW group were found positive for the virus by real-time RT-PCR screening of heart tissue, but only one showed clinical signs of ISA. None of the control fish tested positive for ISA virus RNA. The results for the screening of ISA virus RNA in the seawater experiment and the results of the challenge in Atlantic Salmon are summarized in Table 2. The lowest *Ct*-value obtained in fish (heart tissue) in the challenge experiment was 12.9. This was an individual salmon challenged with a water sample collected after 12 h exposure (T12) of the virus to SSW. The ISA virus segment six, obtained from heart tissue, was sequenced and identified as identical to the segment six sequence of the ISA virus added to the seawater beakers (accession number: KC905164).

DISCUSSION

Waterborne transmission of ISA virus between Atlantic Salmon production sites in seawater has been portrayed as a major risk factor for dissemination of this virus (Vagsholm et al. 1994; Jarp and Karlsen 1997; McClure et al. 2005b; Gustafson et al. 2007; Scheel et al. 2007; Lyngstad et al. 2008, 2011; Mardones et al. 2009, 2011; Aldrin et al. 2010, 2011; Murray et al. 2010). With the exception of Lyngstad et al. (2011) this

TABLE 2. Results of the screening of ISA virus RNA in the seawater and results of the infectivity experiment in Atlantic Salmon. The screening was done at the sampling times listed in the table and from the four different physical conditions (SSW = sterile seawater, SSW+UV = sterile seawater with UV, NSW = normal seawater, NSW+UV = normal seawater with UV). Presence of ISA virus RNA is presented as *Ct*-values (*Ct* = cycle threshold for the real-time RT-PCR screening of the water samples. The results from infectivity experiment are presented as number of fish tested positive (i.e., positive fish/total fish) for ISA virus by real-time RT-PCR in heart tissue (– = tested negative).

	SSW		NSW		SSW+UV		NSW+UV	
Sampling time	*Ct*-values water	Positive fish	*Ct*-values water	Positive fish	*Ct*-values water	Positive fish	*Ct*-values water	Positive fish
T0	20.69		20.74		19.81		21.26	
T3	20.32	4/5	21.08	–	20.63	–	22.62	–
T6	21.55	4/5	22.22	–	22.35	–	24.02	–
T9	20.76	4/5	22.17	–	21.69	–	23.65	–
T12	19.72	2/5	21.47	–	21.66	–	21.66	–
T24	19.89	–	23.93	–	22.80	–	26.21	–
T48	21.18	–	27.01	–	26.30	–	30.87	–
T72	20.46	–	28.20	–	27.78	–	32.58	–

assumption is mainly based on challenge experiments demonstrating transmission of the virus between individuals within tanks, survival of ISA virions in sterile freshwater and seawater, and observations or modeling of more than one outbreak in local areas within a short time span (so called "hot spots"). Sequencing of ISA virus in such hot spots may, in some cases, lead to the conclusion that the viruses are related and in other cases are not (Nylund et al. 2006; Lyngstad et al. 2011; Plarre et al. 2012). However, there are no studies on the survival of ISA virions in natural seawater under exposure to UVR, i.e., a simulation of natural conditions. Several studies discuss the importance of waterborne transmission as a risk factor for spreading ISA virus between marine production sites assuming that the ISA virus remains infectious in seawater for an appropriate period (Vagsholm et al. 1994; Jarp and Karlsen 1997; Thorud and Håstein 2003; Gustafson et al. 2005, 2007; McClure et al. 2005a; Scheel et al. 2007; Lyngstad et al. 2008, 2011; Mardones et al. 2009, 2011; Aldrin et al. 2010, 2011; Murray et al. 2010). The sources most referred to when it comes to survival of ISA virions are Nylund et al. (1994), Torgersen (1997), Rimstad and Mjaaland (2002), Rimstad et al. (2007), and MacLeod et al. (unpublished). However, the actual data presented in these studies to support the assumption that ISA virions can keep their infectivity for a several hours in seawater are not conclusive, and none of these studies included an experimental design that can be reproduced, with the exception of Nylund et al. (1994). Nevertheless, the latter study says nothing about survival in seawater only since the seawater used was diluted 1:1 with ISA virus-infected blood. This makes the present study the first attempt to test survival of ISA virus under simulated natural conditions.

The main result of our study was that the survival time of ISA virions in natural seawater was very limited compared with what has previously been reported (Nylund et al. 1994; Rimstad and Mjaaland 2002; Rimstad et al. 2007; MacLeod et al., unpublished). Infective ISA virions were not present 3 h after exposure to natural seawater despite positive real-time RT-PCR results showing the presence of inactivated ISA virus. Infective virions were still present in sterile seawater 12 h after the start of the experiment. The infectivity was lost within 3 h in sterile seawater exposed to UVR, which showed that the effect of UVR at a level that is naturally present in the open sea also reduced the presence of infectious ISA virions at a rate similar to that observed in natural seawater without UVR.

The exact end points for the survival of ISA virions under the different conditions in this study are not known. However, in this experiment the survival time was less than 3 h when the virions were in natural seawater without UVR or when exposed to UVR in either sterile or natural seawater. Thus, the survival time for infectious ISA virions in sterile seawater alone must be less than 24 h. A major variable factor in survival studies, as presented in this paper, is of course the biological activity in seawater, which varies throughout the day in addition to variation throughout the year. The biological activity in seawater is much lower during winter in the North Atlantic (Norwegian coast) than it is during the period from March to October, which includes periods with algal blooms. These algal blooms have a profound effect on the whole microbial food web. The lysis of bacteria and phytoplankton by infectious viruses should be especially emphasized in this context (Bratbak et al. 1994). Viruses are therefore not only crucial for the functioning of the lower levels of the marine ecosystems, but also for playing a key regulatory role in geochemical cycling and food web dynamics (Thingstad and Lignell 1997). Increased microbial activity, including viral lysis, can lead to enhanced levels of dissolved DNA and RNA together with protein fragments, which seem to be stimulatory with respect to the formation of extracellular nucleases and proteases (Karl and Bailiff 1989; Maruyama et al. 1993; Tsai et al. 1995; Manachini and Fortina 1998). In addition

to the seasonal variation in virus concentrations, Jiang and Paul (1994) found that in the case of DNA viruses, abundance is also positively correlated with temperature and negatively correlated with salinity. Temperature can also have a profound effect on the infectivity of ISA virions (Falk et al. 1997), while the observed impact of salinity is limited to one study using sterile freshwater and seawater (Tapia et al. 2013). The majority of the ISA outbreaks in Norway occur in the spring–autumn period at temperatures between 10°C and 15°C (Thorud 1991), i.e., in the period with the most intensive UVR and highest biological activity in the sea. The highest shedding of ISA virions occurs during this period when the salmon are experiencing ISA, whereas the production of virions from carriers that are present in the sea during winter is probably limited.

As shown in this study, UVR is an important environmental factor that could limit survival and spreading of viruses (Onji et al. 2000; this study). Typical for the summer period for wavelengths of 305, 320, 340, and 380 nm are 1% UVR depths of 2–4, 3.5–6, 5–8, and 9–15 m, respectively, in open coastal waters of western Norway (Erga et al. 2005). The presence of UV-absorbing colored dissolved organic matter (CDOM) will reduce the UV penetration depths farther during the summer and autumn. It is therefore reasonable to believe that the survival time for ISA virions in western Norwegian coastal waters will be influenced both by microbial activity resulting in enhanced RNase and protease levels and UV levels. These environmental factors are supposed to vary with season, latitude, and depth. Similar variation in survival time is also expected to occur along the Chilean coast although the variation throughout the year is probably less than in Norwegian waters due to latitudinal effects. According to Tedetti and Sempere (2006) winter UV-light penetration depths on the central coast of Chile are on a scale 5–10 times deeper than for western Norwegian fjord waters and about 1–2 times deeper than for western Norwegian coastal water (Erga et al. 2005).

It has been suggested that ISA virions drift between sites attached to lipid films in the sea surface. This lipid film can, for example, be due to leakage from feed pellets or "fat-gulping" by the fish, and is often seen close to fish farming sites. However, the presence of a surface lipid film is not supposed to have an influence on UV absorption since lipid absorbs light from the infrared part of the spectrum (Weissleder and Ntziachristos 2003). This means that sunlight should be able to destroy the infectivity of ISA virions in such surface lipid films.

There is great variability spatially and temporally in surface current speed (5 m deep) in salmon farming areas. Some areas have typical current speeds ranging from 1 to 10 cm/s, while in more exposed sites the average current speed may exceed >40 cm/s (Ingrid Ellingsen, SINTEF, personal communication). Provided that the current is stable in one direction from one farming site to the next it would take approximately 8 h at a speed of 10 cm/s and approximately 2 h if the speed is 40 cm/s to cover a distance of 3.0 km. The concentration of waterborne virions will also be diluted during transport from the infected site (Oye and Rimstad 2001). This dilution effect will reduce the chance for an infective dose of ISA virions to reach Atlantic Salmon in neighboring farms (Gregory et al. 2009). Hence, the effect of UVR and biological activity in natural seawater found in the present study, combined with the dilution effect and temperature during the major outbreak periods, means that passive horizontal transmission in seawater over long distance is not very likely.

Based on the available literature and the present study, a distance of 2.5 km between marine production sites for Atlantic Salmon used in the present management of salmon farms in Norway and Chile should, in most cases, be enough to prevent passive transmission of the virus through seawater. It cannot be excluded that ISA virus could be transmitted between marine productions sites via vectors (possibly caligids or escaped Atlantic Salmon) or human activity (e.g., well-boat transport of infected smolts, movement of equipment), which means that the industry should continue to focus on these aspects during the marine production phase. However, previous studies suggest that the ISA virus can be transmitted vertically from broodfish to embryos (Søfteland 2005; Nylund et al. 2006; Vike et al. 2009; Plarre et al. 2012), and it is our opinion that to achieve a further reduction in the number of ISA outbreaks in Chile and Norway, salmon farmers will also have to focus on securing an ISA virus-free source of broodfish.

ACKNOWLEDGMENTS

The authors thank Stian Nylund, Pharmaq Analytiq, Bergen, Norway, for their contribution to some of the analysis. Also thanks to Oddvar Repstad, EWOS Innovation, Norway, for helping with the sampling of fish.

REFERENCES

Aldrin, M., T. M. Lysngtad, A. B. Kristoffersen, B. Storvik, O. Borgan, and P. A. Jansen. 2011. Modelling the spread of infectious salmon anaemia among salmon farms based on seaway distances between farms and genetic relationships between infectious salmon anaemia virus isolates. Journal of the Royal Society Interface 8:1346–1356.

Aldrin, M., B. Storvik, A. Frigessi, H. Viljugrein, and P. A. Jansen. 2010. A stochastic model for the assessment of the transmission pathways of heart and skeleton muscle inflammation, pancreas disease and infectious salmon anaemia in marine fish farms in Norway. Preventive Veterinary Medicine 93:51–61.

Andersen, L., K. Hodneland, and A. Nylund. 2010. No influence of oxygen levels on pathogenesis and virus shedding in salmonid alphavirus (SAV)-challenged Atlantic Salmon (*Salmo salar* L.). Virology Journal [online serial] 7:198. DOI: 10.1186/1743-422X-7-198.

Bouchard, D. A., K. Brockway, C. Giray, W. Keleher, and P. L. Merrill. 2001. First report of infectious salmon anemia (ISA) in the United States. Bulletin of the European Association of Fish Pathologists 21:86–88.

Bratbak, G., F. Thingstad, and M. Heldal. 1994. Viruses and the microbial loop. Microbial Ecology 28:209–221.

Cottet, L., M. C. S. Martin, M. Tello, E. Olivares, A. Rivas-Aravena, E. Vallejos, A. M. Sandino, and E. Spencer. 2010. Bioinformatic analysis of the genome of infectious salmon anemia virus associated with outbreaks with high mortality in Chile. Journal of Virology 84:11916–11928.

Cunningham, C. O., A. Gregory, J. Black, I. Simpson, and R. S. Raynard. 2002. A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bulletin of the European Association of Fish Pathologists 22:366–374.

Dannevig, B. H., K. Falk, and E. Namork. 1995. Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cellline from Atlantic Salmon head kidney. Journal of General Virology 76:1353–1359.

Devold, M., K. Falk, O. B. Dale, B. Krossoy, E. Biering, V. Aspehaug, F. Nilsen, and A. Nylund. 2001. Strain variation, based on the hemagglutinin gene, in Norwegian ISA virus isolates collected from 1987 to 2001: indications of recombination. Diseases of Aquatic Organisms 47:119–128.

Devold, M., M. Karlsen, and A. Nylund. 2006. Sequence analysis of the fusion protein gene from infectious salmon anemia virus isolates: evidence of recombination and reassortment. Journal of General Virology 87:2031–2040.

Devold, M., B. Krossoy, V. Aspehaug, and A. Nylund. 2000. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier Sea Trout *Salmo trutta* after experimental infection. Diseases of Aquatic Organisms 40:9–18.

Duesund, H., S. Nylund, K. Watanabe, K. F. Ottem, and A. Nylund. 2010. Characterization of a VHS virus genotype III isolated from Rainbow Trout (*Oncorhynchus mykiss*) at a marine site on the west coast of Norway. Virology Journal [online serial] 7:19. DOI: 10.1186/1743-422X-7-19.

Erga, S. R., K. Aursland, O. Frette, B. Hamre, K. K. Lotsberg, J. J. Stamnes, J. Aure, F. Rey, and K. Stamnes. 2005. UV transmission in Norwegian marine waters: controlling factors and possible effects on primary production and vertical distribution of phytoplankton. Marine Ecology Progress Series 305:79–100.

Falk, K., E. Namork, E. Rimstad, S. Mjaaland, and B. H. Dannevig. 1997. Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic Salmon (*Salmo salar* L.). Journal of Virology 71:9016–9023.

Godoy, M. G., A. Aedo, M. J. T. Kibenge, D. B. Groman, C. V. Yason, H. Grothusen, A. Lisperguer, M. Calbucura, F. Avendano, M. Imilan, M. Jarpa, and F. S. B. Kibenge. 2008. First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic Salmon (*Salmo salar*) in Chile. BMC Veterinary Research [online serial] 4:28. DOI: 10.1186/1746-6148-4-28.

Gregory, A., L. A. Munro, M. Snow, K. L. Urquhart, A. G. Murray, and R. S. Raynard. 2009. An experimental investigation on aspects of infectious salmon anaemia virus (ISAV) infection dynamics in seawater Atlantic Salmon, *Salmo salar* L. Journal of Fish Diseases 32:481–489.

Gustafson, L. L., S. K. Ellis, and C. A. Bartlett. 2005. Using expert opinion to identify risk factors important to infectious salmon-anemia (ISA) outbreaks on salmon farms in Maine, USA and New Brunswick, Canada. Preventive Veterinary Medicine 70:17–28.

Gustafson, L. L., S. K. Ellis, M. J. Beattie, B. D. Chang, D. A. Dickey, T. L. Robinson, F. P. Marenghi, P. J. Moffett, and F. H. Page. 2007. Hydrographics and the timing of infectious salmon anemia outbreaks among Atlantic Salmon (*Salmo salar* L.) farms in the Quoddy region of Maine, USA and New Brunswick, Canada. Preventive Veterinary Medicine 78:35–56.

Hovland, T., A. Nylund, K. Watanabe, and C. Endresen. 1994. Observation of infectious salmon anenia virus in Atalantic Salmon, *Salmo Salar* L. Journal of Fish Diseases 17:291–296.

Jarp, J., and E. Karlsen. 1997. Infectious salmon anaemia (ISA) risk factors in sea-cultured Atlantic Salmon *Salmo salar*. Diseases of Aquatic Organisms 28:79–86.

Jiang, S. C., and J. H. Paul. 1994. Seasonal and diel abundance of viruses and occurrence of lysogeny/bacteriocinogeny in the marine-environment. Marine Ecology Progress Series 104:163–172.

Jones, S. R. M., and D. B. Groman. 2001. Cohabitation transmission of infectious salmon anemia virus among freshwater-reared Atlantic Salmon. Journal of Aquatic Animal Health 13:340–346.

Karl, D. M., and M. D. Bailiff. 1989. The measurement and distribution of dissolved nucleic-acids in aquatic environments. Limnology and Oceanography 34:543–558.

Kibenge, F. S. B., O. N. Garate, G. Johnson, R. Arriagada, M. J. T. Kibenge, and D. Wadowska. 2001. Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho Salmon in Chile. Diseases of Aquatic Organisms 45:9–18.

Kibenge, F. S. B., M. G. Godoy, Y. Wang, M. J. T. Kibenge, V. Gherardelli, S. Mansilla, A. Lisperger, M. Jarpa, G. Larroquete, F. Avendano, M. Lara, and A. Gallardo. 2009. Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virology Journal [online serial] 6:88. DOI: 10.1186/1743-422X-6-88.

King, A. M. Q., M. J. Adams, E. B. Carstens, and E. J. Lefkowitz. 2012. Virus taxonomy: classification and nomenclature of virus. Ninth report of the International Committee on Taxonomy of Virus. Elsevier Academic Press, San Diego, California.

Koren, C. W. R., and A. Nylund. 1997. Morphology and morphogenesis of infectious salmon anaemia virus replicating in the endothelium of Atlantic Salmon *Salmo salar*. Diseases of Aquatic Organisms 29:99–109.

Krossoy, B., I. Hordvik, F. Nilsen, A. Nylund, and C. Endresen. 1999. The putative polymerase sequence of infectious salmon anemia virus suggests a new genus within the Orthomyxoviridae. Journal of Virology 73:2136–2142.

Lovdal, T., and O. Enger. 2002. Detection of infectious salmon anemia virus in sea water by nested RT-PCR. Diseases of Aquatic Organisms 49:123–128.

Lovely, J. E., B. H. Dannevig, K. Falk, L. Hutchin, A. M. MacKinnon, K. J. Melville, E. Rimstad, and S. G. Griffiths. 1999. First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. Diseases of Aquatic Organisms 35:145–148.

Lyngøy, C. 2002. Infectious salmon anemia in Norway and the Faroe Islands: an industrial approach. Pages 97–109 *in* O. Miller and R. C. Cipriano, editors. International response to infectious salmon anemia: prevention, control, and eradication. U.S. Department of Agriculture, Animal and Plant Health Inspection Service, U.S. Geological Survey, National Marine Fisheries Service, Technical Bulletin 1902, Washington, D.C.

Lyngstad, T. M., M. J. Hjortaas, A. B. Kristoffersen, T. Markussen, E. T. Karlsen, C. M. Jonassen, and P. A. Jansen. 2011. Use of Molecular epidemiology to trace transmission pathways for infectious salmon anaemia virus (ISAV) in Norwegian salmon farming. Epidemics 3:1–11.

Lyngstad, T. M., P. A. Jansen, H. Sindre, C. M. Jonassen, M. J. Hjortaas, S. Johnsen, and E. Brun. 2008. Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003–2005. Preventive Veterinary Medicine 84:213–227.

Manachini, P. L., and M. G. Fortina. 1998. Production in sea-water of thermostable alkaline proteases by a halotolerant strain of *Bacillus licheniformis*. Biotechnology Letters 20:565–568.

Mardones, F. O., A. M. Perez, and T. E. Carpenter. 2009. Epidemiologic investigation of the re-emergence of infectious salmon anemia virus in Chile. Diseases of Aquatic Organisms 84:105–114.

Mardones, F. O., A. M. Perez, P. Valdes-Donoso, and T. E. Carpenter. 2011. Farm-level reproduction number during an epidemic of infectious salmon anemia virus in southern Chile in 2007–2009. Preventive Veterinary Medicine 102:175–184.

Maruyama, A., M. Oda, and T. Higashihara. 1993. Abundance of virus-sized non-dnase-gigestible DNA (coated DNA) in eutrophic seawater. Applied and Environmental Microbiology 59:712–717.

McClure, C., K. Hammell, and I. Dohoo. 2005a. Risk factors for outbreaks of infectious salmon anemia in farmed Atlantic Salmon, *Salmo salar*. Preventive Veterinary Medicine 72:263–280.

McClure, C. A., K. L. Hammell, H. Stryhn, I. R. Dohoo, and L. J. Hawkins. 2005b. Application of surveillance data in evaluation of diagnostic tests for infectious salmon anemia. Diseases of Aquatic Organisms 63:119–127.

Mjaaland, S., E. Rimstad, K. Falk, and B. H. Dannevig. 1997. Genomic characterization of the virus causing infectious salmon anemia in Atlantic Salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost. Journal of Virology 71:7681–7686.

Muller, P. Y., H. Janovjak, A. R. Miserez, and Z. Dobbie. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques 32:1372–1374, 1376, 1378–1379.

Mullins, J. E., D. Groman, and D. Wadowska. 1998. Infectious salmon anaemia in salt water Atlantic Salmon (*Salmo salar* L.) in New Brunswick, Canada. Bulletin of the European Association of Fish Pathologists 18:110–114.

Murray, A. G., L. A. Munro, I. S. Wallace, B. Berx, D. Pendrey, D. Fraser, and R. S. Raynard. 2010. Epidemiological investigation into the re-emergence and control of an outbreak of infectious salmon anaemia in the Shetland Islands, Scotland. Diseases of Aquatic Organisms 91:189–200.

Nylund, A., M. Devold, H. Plarre, E. Isdal, and M. Aarseth. 2003. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. Diseases of Aquatic Organisms 56:11–24.

Nylund, A., T. Hovland, K. Hodneland, F. Nilsen, and P. Lovik. 1994. Mechanisms for transmission of infectitous salmon anemia (ISA). Diseases of Aquatic Organisms 19:95–100.

Nylund, A., H. Plarre, M. Karlsen, F. Fridell, K. F. Ottem, A. Bratland, and P. Saether. 2006. Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Archives of Virology 152:151–179.

Nylund, A., C. Wallace, and T. Hovland. 1993. The possible role of *Lepeophtheirus salmonis* (Kroyer) in the transmission of infectious salmon anaemia. Pages 367–373 *in* G. A. Boxshall and D. Defaye, editor. Pathogens of wild and farmed fish: sea lice. Ellis Horwood, Chichester, UK.

Olsvik, P. A., K. K. Lie, A. E. O. Jordal, T. O. Nilsen, and I. Hordvik. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic Salmon. BMC Molecular Biology [online serial] 6:21. DOI: 10.1186/1471-2199-6-21.

Onji, M., T. Sawabe, and Y. Ezura. 2000. Characteristics of virus like growth suppression agents against phytoplankton obtained from seawater at the mouth of Funka Bay, Hokkaido, Japan. Fisheries Science 66:38–43.

Oye, A. K., and E. Rimstad. 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.

Paul, N. D., and D. Gwynn-Jones. 2003. Ecological roles of solar UV radiation: towards an integrated approach. Trends in Ecology and Evolution 18:48–55.

Plarre, H., M. Devold, M. Snow, and A. Nylund. 2005. Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in western Norway. Diseases of Aquatic Organisms 66:71–79.

Plarre, H., A. Nylund, M. Karlsen, O. Brevik, P. A. Saether, and S. Vike. 2012. Evolution of infectious salmon anaemia virus (ISA virus). Archives of Virology 157:2309–2326.

Rimstad, E., E. Biering, E. Brun, K. Falk, F. Kibenge, S. Mjaaland, M. Snow, and J. R. Winton. 2007. Norwegian Scientific Committee for Food Safety: opinion of the Panel on Animal Health and Welfare. Which risk factors relating to spread of infectious salmon anaemia (ISA) require development of management strategies? Norwegian Scientific Committee for Food Safety, Dok.nr.06/804. Available: http://www.vkm.no/dav/edccd8ceaa.pdf. (January 2014).

Rimstad, E., and S. Mjaaland. 2002. Infectious salmon anaemia virus: an orthomyxovirus causing an emerging infection in Atlantic Salmon. Acta Pathologica Microbiologica et Immunologica Scandinavica 110:273–282.

Ritchie, R. J., M. Cook, K. Melville, N. Simard, R. Cusack, and S. Griffiths. 2001. Identification of infectious salmon anaemia virus in Atlantic Salmon from Nova Scotia (Canada): evidence for functional strain differences. Diseases of Aquatic Organisms 44:171–178.

Rodger, H. D., T. Turnbull, F. Muir, S. Millar, and R. H. Richards. 1998. Infectious salmon anaemia (ISA) in the United Kingdom. Bulletin of the European Association of Fish Pathologists 18:115–116.

Rowley, H. M., S. J. Campbell, W. L. Curran, T. Turnbull, and D. G. Bryson. 1999. Isolation of infectious salmon anaemia virus (ISAV) from Scottish farmed Atlantic Salmon, *Salmo salar* L. Journal of Fish Diseases 22:483–487.

Scheel, I., M. Aldrin, A. Frigessi, and P. A. Jansen. 2007. A stochastic model for infectious salmon anemia (ISA) in Atlantic Salmon farming. Journal of the Royal Society Interface 4:699–706.

Sobsey, M. D., S. E. Oglesbee, and D. A. Wait. 1985. Evaluation of methods for concentrating hepatitis A virus from drinking water. Applied and Environmental Microbiology 50:1457–1463.

Søfteland, E. 2005. Experiments with ISAV infected broodfish (Atlantic Salmon). Can the ISAV be transmitted via gonadal products? Mattilsynet, Universitetet i Bergen, Akvaforsk, Fomas, Veterinærinstituttet, Bolaks, Salmobreed. (Report in Norwegian).

Tapia, E., G. Monti, M. Rozas, A. Sandoval, A. Gaete, and P. Bustos. 2013. Assessment of the *in vitro* of survival of the infectious salmon anaemia (ISAV) under difterent water types and temperature. Bulletin of the European Association of Fish Pathologists 33:3–12.

Tedetti, M., and R. Sempere. 2006. Penetration of ultraviolet radiation in the marine environment: a review. Photochemistry and Photobiology 82:389–397.

Thingstad, T. F., and R. Lignell. 1997. Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquatic Microbial Ecology 13:19–27.

Thorud, K., and H. O. Djupvik. 1988. Infectious anaemia in Atlantic Salmon (*Salmo Salar* L.). Bulletin of the European Association of Fish Pathologists 8:109–111.

Thorud, K. E. 1991. Infectious salmon anemia. Doctoral dissertation. Norwegian College of Veterinary Medicine, Oslo.

Thorud, K. E., and T. Håstein. 2003. Experiences with regulatory responses to infectious salmon anemia in Norway. Pages 155–159 *in* O. Miller and R. C. Cipriano, editors. International response to infectious salmon anemia: prevention, control, and eradication. U.S. Department of Agriculture, Animal and Plant Health Inspection Service, U.S. Geological Survey, National Marine Fisheries Service, Technical Bulletin 1902, Washington, D.C.

Torgersen, Y. 1997. Physican and chemical inactivation of the Infectious salmon anaemia (ISA) virus. Pages 44–53 *in* Proceedings of the workshop on infectious salmon anaemia. New Brunswick Department of Fisheries and Aquaculture, Fredericton, New Brunswick, Canada.

Totland, G. K., B. K. Hjeltnes, and P. R. Flood. 1996. Transmission of infectious salmon anaemia (ISA) through natural secretions and excretions from infected smelts of Atlantic Salmon *Salmo salar* during their presymptomatic phase. Diseases of Aquatic Organisms 26:25–31.

Tsai, Y. L., B. Tran, and C. J. Palmer. 1995. Analysis of viral-RNA persistence in seawater by reverse-transcriptase PCR. Applied and Environmental Microbiology 61:363–366.

Vagsholm, I., H. O. Djupvik, F. V. Willumsen, A. M. Tveit, and K. Tangen. 1994. Infectious salmon anemia (ISA) epidemiology in Norway. Preventive Veterinary Medicine 19:277–290.

Vike, S., S. Nylund, and A. Nylund. 2009. ISA virus in Chile: evidence of vertical transmission. Archives of Virology 154:1–8.

Weissleder, R., and V. Ntziachristos. 2003. Shedding light onto live molecular targets. Nature Medicine 9:123–128.

VETERINARY RESEARCH

RESEARCH Open Access

Low virulent infectious salmon anaemia virus (ISAV) replicates and initiates the immune response earlier than a highly virulent virus in Atlantic salmon gills

Alastair J A McBeath[1*], Yee Mai Ho[1], Maria Aamelfot[2], Malcolm Hall[1], Debes H Christiansen[3], Turhan Markussen[2], Knut Falk[2] and Iveta Matejusova[1]

Abstract

Observations from the field and experimental evidence suggest that different strains of infectious salmon anaemia virus (ISAV) can induce disease of varying severity in Atlantic salmon. Variation in host mortality and dissemination of ISAV isolates with high and low virulence was investigated using immersion challenge; from which mortality, pathological, immunohistochemical and preliminary molecular results have been previously published. Here, real-time RT-PCR analysis and statistical modelling have been used to further investigate variation in virus load and the response of four select immune genes. Expression of type I and II interferon (IFN), Mx and γIFN induced protein (γIP) to high and low pathogenic virus infection were examined in gill, heart and anterior kidney. In addition, a novel RNA species-specific assay targeting individual RNA types was used to investigate the separate viral processes of transcription and replication. Unexpectedly, the low virulent ISAV (LVI) replicated and transcribed more rapidly in the gills compared to the highly virulent virus (HVI). Subsequently LVI was able to disseminate to the internal organs more quickly and induced a more rapid systemic immune response in the host that may have offered some protection. Contrary to this, HVI initially progressed more slowly in the gills resulting in a slower generalised infection. However HVI ultimately reached a higher viral load and induced a greater mortality.

Introduction

Infectious salmon anaemia virus (ISAV) is an aquatic orthomyxovirus that infects Atlantic salmon. The virus can cause serious disease problems resulting in substantial financial implications for the aquaculture industry. Infectious salmon anaemia (ISA) has occurred annually in Norway [1] since its discovery in 1984 [2] and has also been reported in North America [3-5], Scotland [6,7], Faroe Islands [8,9] and Chile [10]. ISAV is enveloped and possesses a genome of 8 negative-sense single-stranded RNA segments, coding for at least 10 proteins, similar to that of influenza virus [11,12]. Segments 1–4 produce the proteins that comprise the viral ribonucleoprotein core. Segments 5 and 6 encode the fusion (F) [13] and haemagglutinin-esterase (HE) [14,15] surface glycoproteins respectively. Segment 7 possesses two open reading frames (ORFs), the first of which is thought to produce a minor or non-structural protein (NS) [16] that interferes with interferon (IFN) and IFN induced systems [17,18]. The second ORF, generated via splicing, is believed to encode the nuclear export protein (NEP) [19]. Segment 8 also contains two ORFs; ORF1 encodes the major structural matrix (M) protein [14,16] and ORF2 is believed to produce a minor structural protein also with IFN antagonising activity [17]. The M protein is the most abundant protein in the virion [20] and therefore RNA of this segment is readily targeted in diagnostic tests [21].

The pathogenicity of ISAV is a multifactorial trait dependent on the function of viral proteins, interactions with host immune responses as well as various environmental factors. Little is known regarding the specific interplay of these aspects and how they influence ISAV infection. Nevertheless there are multiple reports that

* Correspondence: alastair.mcbeath@scotland.gsi.gov.uk
[1]Marine Scotland Science, Marine Laboratory, Aberdeen, Scotland, UK
Full list of author information is available at the end of the article

different strains of ISAV display different levels of virulence and associated mortality [22-26]. A deletion within the highly polymorphic region (HPR) of segment 6 has been suggested to be an important virulence marker [25,27]. All pathogenic strains reported to date contain an HPR deletion with respect to the putatively ancestral, non-virulent HPR0 virus [9,28]. In addition, substitutions and insertions adjacent to the putative cleavage site in the F protein have also been linked to virulence [27,29]. However, even isolates with identical HPR and F protein sequences have shown variation in virulence [25,27] indicating additional factors are also important.

Vertebrate nucleated cells rapidly secrete the cytokine molecules interferon (IFN) in response to viral infection. In turn, IFNs activate complex signalling pathways inducing the expression of hundreds of direct and indirect antiviral genes [for review see [30,31]]. In fish, both the Type I and Type II IFN systems are now known to be imperative in the innate and adaptive antiviral responses respectively [31,32]. ISAV is a potent inducer of immune genes yet the response offers little protection [25,33,34]. Intraperitoneal injection of a highly virulent ISAV indicated significant monophasic induction of several immune genes in kidney on day 6 post-infection, followed by increased virus production and high mortality [33]. The antiviral protein Mx has become a direct indicator of the innate Type I IFN response, induced by IFNs α and β in a variety of cell types. Similarly, the IFNγ-induced protein (γIP) gene can be used as an indicator of the adaptive Type II IFN response, stimulated by γIFN specifically in immune cells. The expression of these interferon stimulated genes (ISGs) as induced products of each system showed both the innate and adaptive responses were stimulated concurrently by ISAV injection [33]. More recently, cell cultures infected with ISAV of high or low virulence have indicated variations in immune response between strains [35,36].

The present study utilises an immersion infection of Atlantic salmon with two ISAV strains previously classed as low (LVI) and high virulence (HVI) [23,37]. The mortality, pathology, immunohistochemistry (IHC) and initial virus segment 8 qPCR results observed following the challenge are presented in McBeath et al. [37]. Here, the molecular results are analysed in more detail using statistical modelling of the qPCR data of viral segments 7 and 8 obtained from gill, heart and anterior kidney samples. The separate viral processes of replication and transcription were also investigated individually for both viruses using novel RNA species-specific assays based on a recently published method [38]. In addition, the expression levels of four host immune markers were also monitored and statistically analysed to determine if infection with LVI or HVI had variable effects on the immune system. Ultimately, LVI was shown to replicate more rapidly in the gills resulting in more rapid dissemination and immune response throughout the host, yet HVI reached higher viral loads, causing a more serious infection and inducing greater mortality. The results of this study contribute to our understanding of the pathogenesis of this important pathogen in Atlantic salmon aquaculture.

Materials and methods

Virus propagation, immersion challenge and organ sampling

Details regarding the virus production, infection challenge and organ sampling procedure have been described previously [37]. Briefly, fish were challenged by immersion with either 10^4 $TCID_{50}$ of highly virulent ISAV Glesvær 2/90 (HVI) or low virulent Can/F679/99 (LVI), or mock-infected with virus-free cell culture medium as uninfected negative controls. After 2 h, fish were transferred to sampling tanks ($n = 75$) or to observation tanks where cumulative mortalities for each treatment were observed ($n = 20$). Four fish were sampled 6 h post infection (pi) and on days 1 to 8, 10, 12, 14, 19 and 23 pi from each challenge group. Organ samples from gill, heart, and anterior-kidney, were collected in 1 mL RNAlater (Qiagen) and stored at −80 °C.

Viral and immune gene expression real-time RT-PCR

RNA was extracted from 5 mg organ samples (gill, heart, anterior kidney) using the QIAsymphony® RNA robotic system (Qiagen) according to the manufacturers' protocol and eluted in 100 μL RNase-free dH_2O. The RNA was reverse transcribed to cDNA using the TaqMan® Reverse Transcription Reagents kit (Life Technologies, UK) with oligo-$d(T)_{16}$ as described previously [33] using a 20 μL total reaction volume. Real-time RT-PCR assays were performed as described in McBeath et al. [37]. Primer and probe sequences (Additional file 1) for the assays targeting viral gene (no differentiation of ORFs in either assay) segments 7 (seg7) and 8 (seg8) from both European and North-American genogroups, immune markers Type I (α1 and α2), Type II interferon (IFN), Mx, γIFN-induced protein (γIP) and endogenous control elongation factor 1α (ELF), have been described previously [21,33]. Absolute quantitation of transcripts was carried out. The cycle crossing point (Cp) values were converted into expression values normalised against the reference gene, ELF, using the statistical standard curve method to produce relative expression ratios [39].

Statistical analysis

The analysis focussed on modelling the scale of virus loads and immune responses for each ISAV isolate throughout the experiment. Statistical analyses were performed within the R statistical environment version 2.15.2 [40].

All observations greater than zero were transformed to $\log_{10}$, converting the analysis into an evaluation of the variation in the scale of detectable expression for defined genes associated with two ISAV strains over time. The distributions of the transformed observations for each gene-strain-time combination are also more satisfactory approximations of the normal distribution. A preliminary inspection of the transformed observations suggested that there were differences in the dispersion of the scaled values at different times, and weights comprising the reciprocal of the variance for each strain-time combination were therefore calculated. These weights were used for the modelling of the changes in the scale of detectable gene expression for each strain over time using a locally-weighted running-line smoother [41]. Predicted values with their 95% confidence intervals (CI) were plotted. Plots exhibiting minimal overlap of CI for the two strains indicate differences in the trajectory of detectable expression for that gene over time between the two strains. A more formal evaluation of differences between strains was carried out by estimating the reduction in the residual sum of squares of nested linear models [42] describing gene expression on the potential explanatory variables of strain and time; p-values of ≤ 0.05 were categorised as statistically significant.

The association between the difference in the level of expression of the immunological markers (e.g. Mx) of the two strains with the difference in the load of the two strains (evaluated using both the segment 7 and segment 8) was evaluated for each tissue using Spearman's rank correlation coefficient (r_s) [43]. The statistical significance of associations is indicated by "star value" (*$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$).

RNA species specific real-time RT-PCR

RNA species specific reverse transcription (RT) was performed as described previously [38] using tagged primers for mRNA and cRNA (Additional file 1). Reverse transcription was performed at 65 °C using the Thermoscript RT kit (Life Technologies) according to manufacturers' protocols.

Real-time PCR was carried out using SensiFAST Sybr no-rox Master Mix (Bioline) and primer pairs consisting of one primer specific to the tag portion of the RT-tagged primers and the other specific to the appropriate viral sequence (Additional file 1). Reactions consisted of 1 × SensiFAST Sybr no-rox Master Mix, 10 μM each primer, 1 μL cDNA template in a 20 μL volume and were subjected to a 95 °C for 2 min pre-incubation, 45 cycles of 95 °C for 5 s, 62 °C for 10 s, 72 °C for 10 s, followed by melting curve analysis (95 °C for 5 s, 65 °C for 1 min, slow heating to 97 °C and cooling at 40 °C for 30 s) using a Roche Lightcycler LC480. The cycle crossing point (Cp) values were cross checked with melt curve data and samples were deemed positive if the melt curve temperature was within ± 0.5 °C of the expected value. Samples with no Cp value or a Cp value correlated with an incorrect melt curve temperature (i.e. indication of non-specificity) were classed as negative. Samples with dual melt curves were classed as positive if one was at the correct temperature. Due to the complication of non-specificity, no attempt was made to quantify the different RNA types.

Results

Experimental infection and mortality

In fish infected with HVI, mortality began on day 13 pi and the cumulative mortality reached 100% by day 23 pi. In the LVI group, mortality began on day 17 pi and cumulative mortality reached 20% by day 23 pi. All dead fish tested positive in kidney for ISAV segment 8 by real-time PCR. No mortality was observed in the uninfected negative control fish. Further details of the above results including pathology and immunohistochemistry (IHC) are presented in McBeath et al. [37].

Viral kinetics profile by detection of ISAV segment 8

All infected fish tested positive for segment 8 in the gills at every time point. In heart, no virus was detected at 6 h pi, 2/4 fish tested positive for LVI and 1/4 for HVI on day 1 pi. After 2 days, heart was positive in 3/4 fish infected with LVI and 2/4 infected with HVI. Kidney was also negative for segment 8 until day 2 pi when the LVI was detected in all 4 fish and HVI was detected in 2/4 fish. Thereafter from day 3 pi, both viruses were detected in all organ samples. Statistical modelling indicates that the time course of the 2 strains, as indicated by detection of segment 8, differ ($p \leq 0.05$) for all 3 organs (Figure 1). This was most apparent in the early stages of the infection up to day 8 and especially notable in the gills (Figure 1A) during the first 4 days pi. Following day 8 pi, the LVI load almost stabilised in comparison to the HVI load which continued to increase further up to day 15 pi. All uninfected control fish were negative for ISAV.

RNA species specific analysis of segment 8

The assays targeting RNA species corresponding specifically to the replication (cRNA) and transcription (mRNA) processes provided further evidence the LVI had successfully entered and replicated in the gills early in infection (Table 1). The results also suggest HVI has a slower uptake and/or entry into the gills and therefore progresses more slowly into generalised infection. In the LVI group, mRNA was detected in all fish (gills) at day 1 pi and thereafter until experimental end. In comparison, HVI mRNA was not first detected in gills until day 4 pi (in 3 fish) which correlated with the first large increase in HVI load

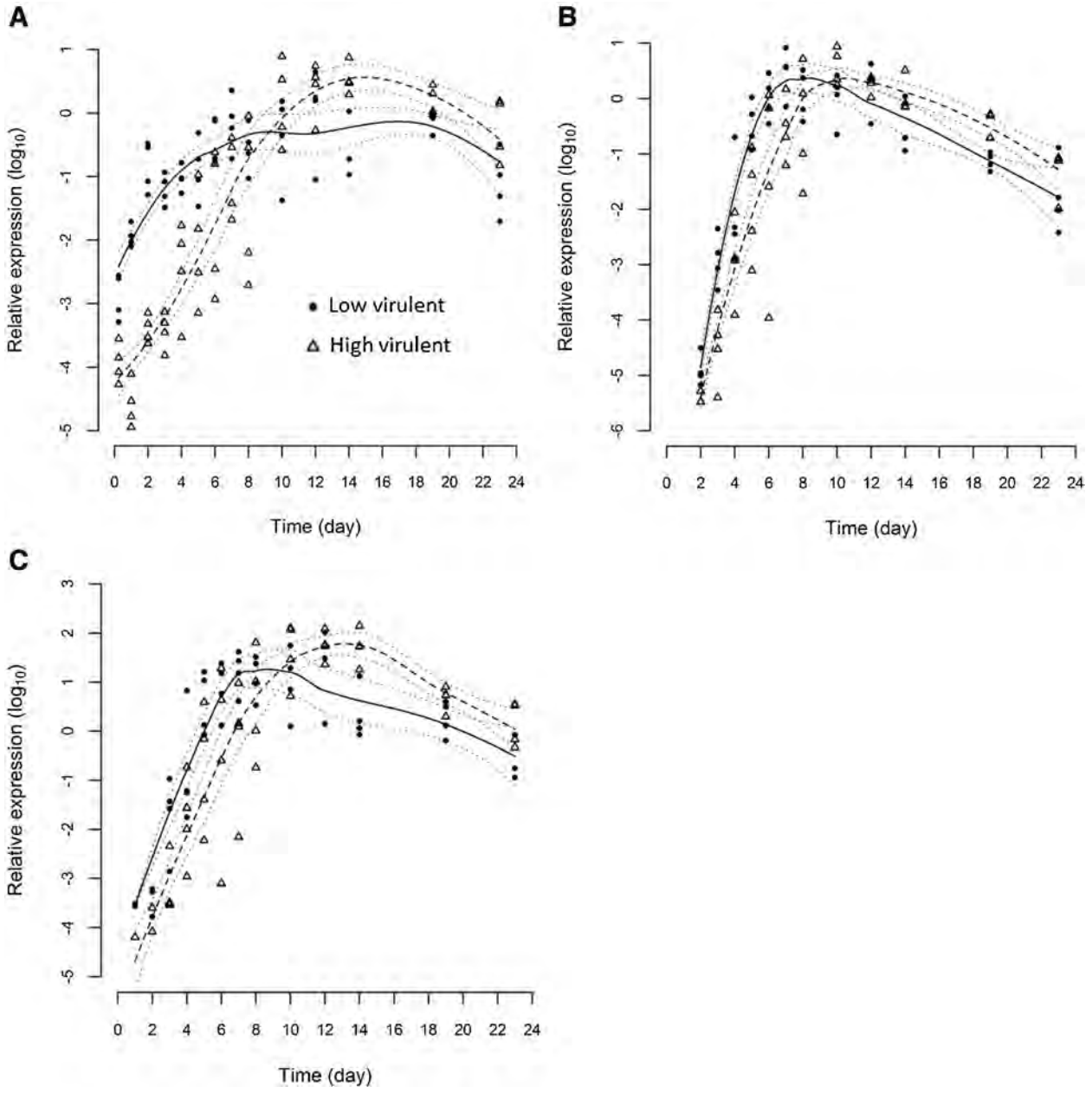


Figure 1 Statistical modelling of ISAV segment 8. Statistical modelling of viral segment 8 total RNA load profiles of high virulent (dashed line) and low virulent (solid line) strains measured by real-time RT-PCR from gill **(A)**, anterior kidney **(B)** and heart **(C)** tissues post-immersion infection at Day 0. Dotted lines indicate 95% confidence intervals.

by seg8 qPCR. Similarly, the LVI cRNA was also detected earlier in the gills than in the HVI group, although there was increased variation at the individual level in the latter. The difference in mRNA and cRNA production between strains was less pronounced in the kidney and the heart. In kidney, mRNA of both ISAV strains was detected at day 3 pi, however the number of positive individuals remained variable in the HVI group until day 7 pi. The first detection of cRNA in the kidney of the LVI group was in 1 fish on day 3 pi, followed by 3 fish on day 4 pi. The HVI cRNA was first detected in the kidney from 3 fish on day 4 pi (on days 5 and 6 also). In heart, the first mRNA detections for both viruses were from single fish on days 2 and 4 respectively, prior to all 4 fish on days 4 and 7 respectively. The first cRNA detections in heart occurred in 1 fish on day 3 pi in the LVI group and in 2 fish on day 4 in the HVI group.

As expected, the Cp values indicated cRNA was present in lower amounts than mRNA [38,44]. Cross-checking Cp results with melt curve data indicated non-specificity

Table 1 RNA strand specific real-time RT-PCR

	Gill mRNA		Gill cRNA		Kidney mRNA		Kidney cRNA		Heart mRNA		Heart cRNA	
Day	LVI	HVI	LVI	HVI	LVI	HVI	LVI	HVI	LVI	HVI	LVI	HVI
0.25	0	0	0	0	0	0	0	0	na	na	na	na
1	4	0	1	0	0	0	0	1	0	0	0	0
2	4	0	2	0	0	0	0	0	1	0	0	0
3	4	0	3	1	4	2	1	0	3	0	1	0
4	4	3	2	1	4	4	3	3	4	1	2	2
5	4	3	3	2	4	3	4	3	4	2	4	3
6	4	4	4	2	4	3	4	3	4	3	4	3
7	4	4	4	3	4	4	4	4	4	4	4	4
8	4	4	4	2	4	4	4	4	na	na	na	na
10-23	4	4	4	4	4	4	4	4	na	na	na	na

Number of fish (max. = 4) testing positive for mRNA or cRNA of low virulent (LVI) or highly virulent (HVI) virus using RNA strand specific real-time RT-PCR analysis of gill, anterior kidney and heart. na = not applicable.

interfered with the mRNA assay when template levels were low (seg8 Cp > 32), as discussed previously [38]. Contrary to this, the cRNA assay was more specific, even at very low levels (seg8 Cp > 38) (data not shown).

Immune gene expression

The expression of four immune markers, Type I and II IFN, Mx and γIP, was measured in gills, heart and anterior kidney to provide information on both innate and adaptive immune responses upon infection with the two different ISAV strains. All immune genes in infected fish were strongly up-regulated in comparison to the negative control fish throughout the experiment (Table 2). The maximum increase in transcript levels for all four immune gene markers to both ISAV strains was higher in kidney and heart compared to gills. The highest maximum increase was observed for Type II IFN in kidney. Statistical modelling indicates that the time course of expression for all four genes differ between the two strains ($p \leq 0.05$) in gill (Figure 2), anterior kidney (Figure 3) and heart (Figure 4) indicating a differential immunological host response to the two viruses. In all cases, the immune genes were stimulated significantly more in the LVI group than the HVI group up to day 7 or 8 pi. In contrast, after day 8, the immune genes were expressed at higher levels in the HVI fish.

Segment 8 and immune gene correlation analysis

The associations between the difference in virus segment 8 of the low and the highly virulent viruses and the difference in immune gene expression response to each strain were investigated using correlation. The rationale for this is that a positive association between an increasing difference in load between the two strains and an increasing immune response is indicative of a possible causal relationship between viral load and immune response. Association of the differences between the segment 8 viral load and the expression of all immune genes in the gill and heart for both LVI and HVI (Table 3) were observed ($p \leq 0.05$), indicating an association between viral load as measured by segment 8 RNA quantity and immune gene expression in these organs.

Segment 7

Expression profiles for genomic segment 7 in gill, kidney and heart (Figure 5) were similar overall to those of segment 8, although a higher quantity of segment 7 was produced by LVI than HVI up to day 8 pi. This was

Table 2 Fold increase in expression of immune genes

	Gill			Kidney			Heart		
Gene	LVI	HVI	Control	LVI	HVI	Control	LVI	HVI	Control
Type I IFN	7.7	**10.4**	2.7	**49.6**	24.6	2.4	23.6	**100.5**	1.8
Mx	**71.6**	63	1.4	**213.4**	100	2.9	257.2	**271.9**	2.6
Type II IFN	30.2	**47**	5.9	**1443.2**	485	6.4	**394.5**	197.9	7.6
gIP	**93.2**	54	2.3	242.5	**255**	4.1	290.7	**424.3**	9.8

Fold increase from 6 h to the maximum peak of immune gene expression (on any given day) post-infection (pi) for fish infected with ISAV of either low (LVI) or high (HVI) virulence, or cell culture media to serve as negative controls. Figures in bold indicate the highest fold-induction for each gene in each organ.

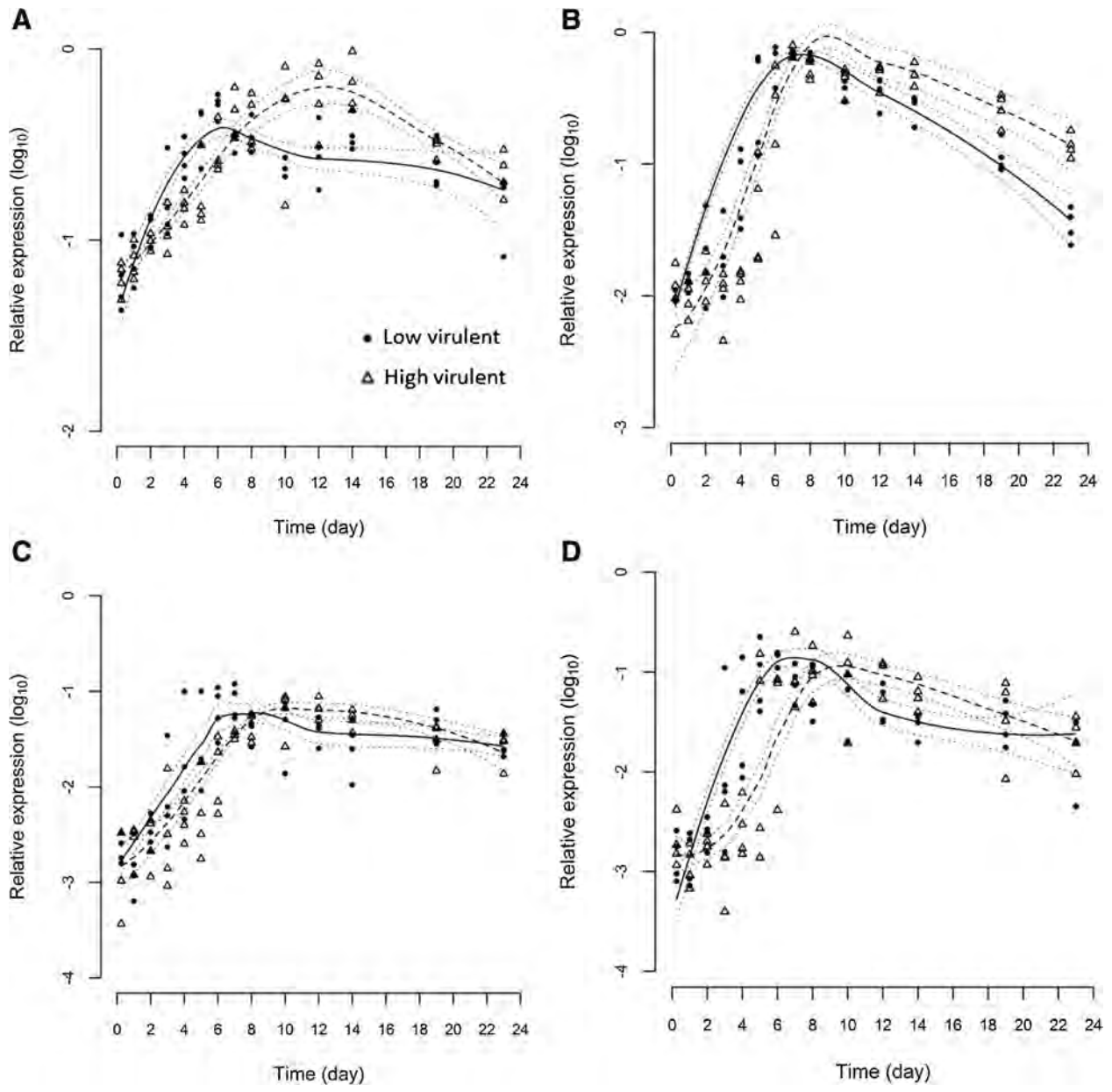


Figure 2 Statistical modelling of immune genes in gill. Statistical modelling of 4 immune marker expression profiles, Type I IFN **(A)**, Mx **(B)**, Type II IFN **(C)** and γIP **(D)** in gills of fish infected with high virulent (dashed line) or low virulent (solid line) strains measured by real-time RT-PCR. Dotted lines indicate 95% confidence intervals.

more evident upon comparing a ratio of the two segments for each strain (Additional file 2). Following day 8 pi, unlike segment 8, there was little difference between the two viruses with regards to segment 7 RNA production as indicated by overlapping confidence intervals. In contrast to the results for segment 8, there was no evidence of an association in differences between the segment 7 viral loads and differences in immune response for gill and heart, although positive correlation ($p \leq 0.05$) was observed in kidney (Table 3).

Discussion

In the present study, organs from fish infected with low virulent ISAV (LVI) exhibited a higher viral load in the first 8 days of the infection compared to the highly virulent virus (HVI). This was most prominent in the gills during the first 4 days post-infection. The rapid increase in LVI segment 8 RNA load suggests the occurrence of a fast primary replication phase in the gill. The RNA species specific assays provided further support for this, indicating the LVI successfully gained fast entry to host

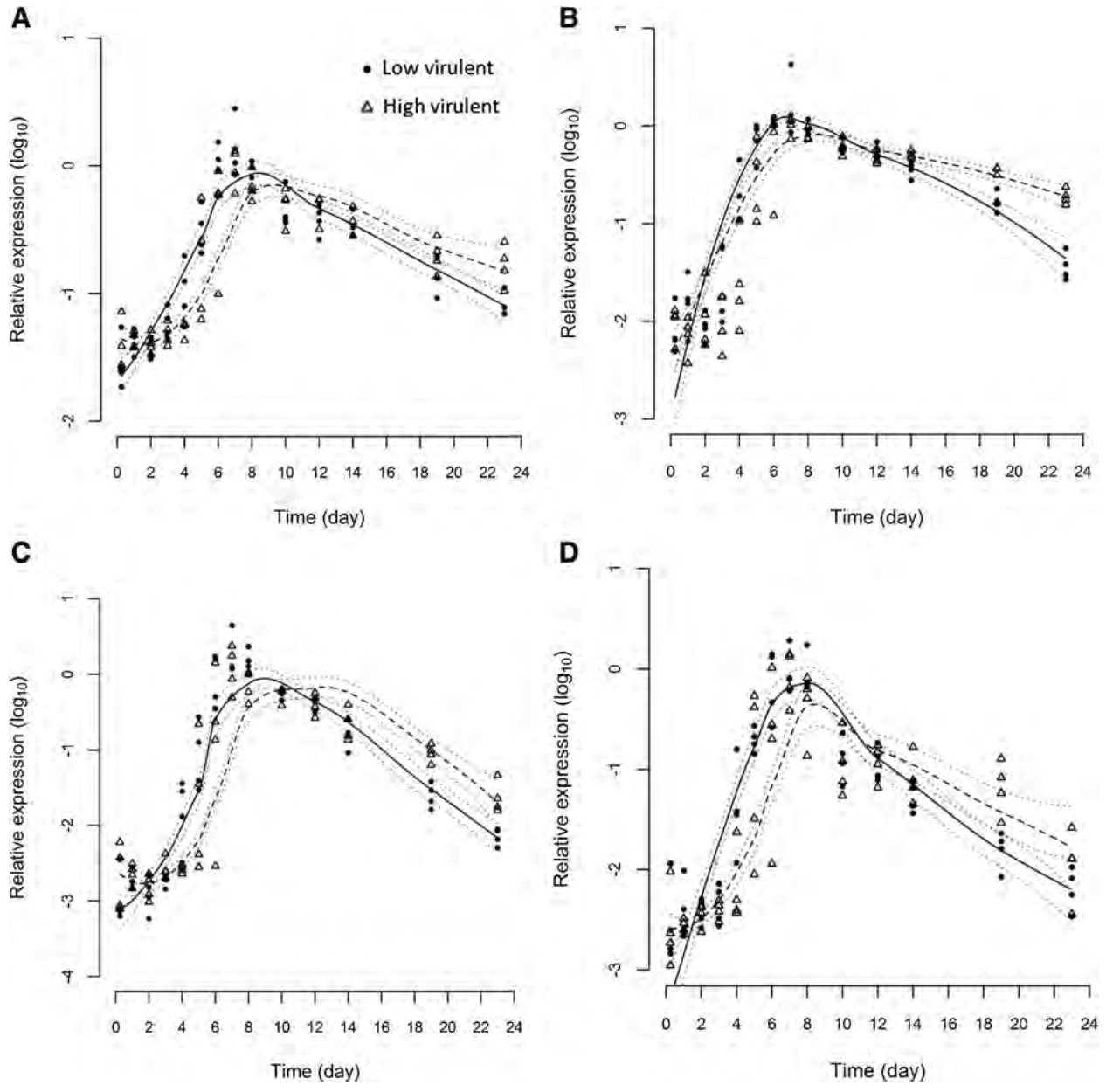


Figure 3 Statistical modelling of immune genes in kidney. Statistical modelling of 4 immune marker expression profiles, Type I IFN **(A)**, Mx **(B)**, Type II IFN **(C)** and γIP **(D)** in anterior kidney of fish infected with high virulent (dashed line) or low virulent (solid line) strains measured by real-time PCR. Dotted lines indicate 95% confidence intervals.

gill cells, and was both actively replicating and transcribing in this early stage of infection. All four immune genes, representing both Type I and Type II IFN systems and two products thereof, were significantly differentially expressed when comparing the two ISAV isolates. This suggested differences in the host response towards the two virus infections. Statistical correlations provided further evidence suggesting a causal link between virus production (as measured by segment 8 RNA) and up-regulation of the studied immune genes in the gill and heart.

In an immersion challenge, all mucosal surfaces, including gill, skin, eye and gut, are possible entry points for microbial infections. The gills have previously been suggested as the main entry port for ISAV [45]. However fin and skin have both been implicated as important entry points for viral haemorrhagic septicaemia (VHSV), infectious haematopoietic necrosis virus (IHNV) and koi

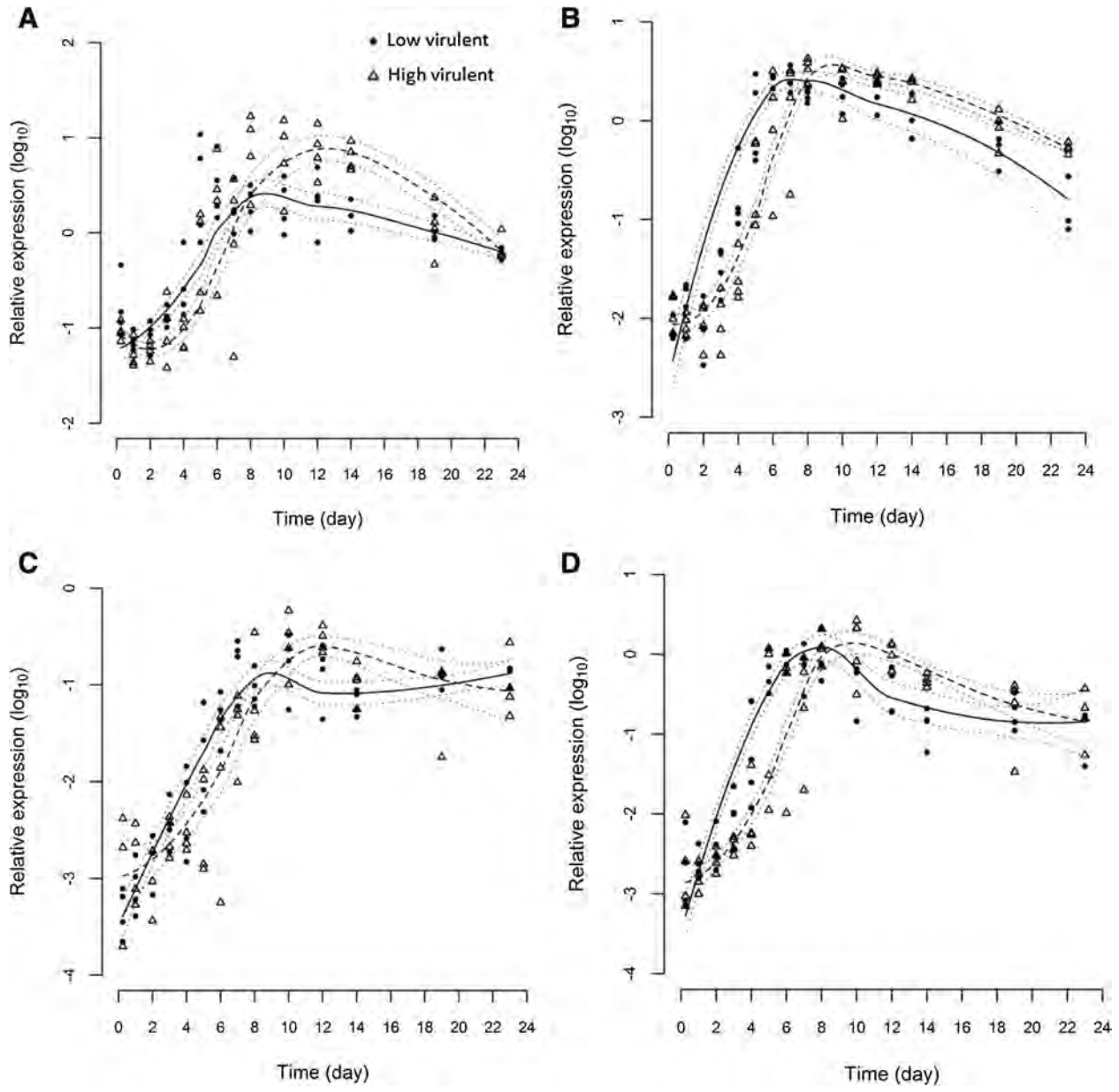


Figure 4 Statistical modelling of immune genes in heart. Statistical modelling of 4 immune marker expression profiles, Type I IFN **(A)**, Mx **(B)**, Type II IFN **(C)** and γIP **(D)** in heart of fish infected with high virulent (dashed line) or low virulent (solid line) strains measured by real-time RT-PCR. Dotted lines indicate 95% confidence intervals.

Table 3 Correlation analysis

	Segment 8			Segment 7		
Immune gene	**Gill**	**Kidney**	**Heart**	**Gill**	**Kidney**	**Heart**
Mx	0.75**	0.9***	0.9***	0.23	0.72**	0.12
γIP	0.61*	0.43	0.96***	−0.002	0.46	0.12
Type I IFN	0.61*	0.43	0.86***	−0.002	0.56*	−0.002
Type II IFN	0.82***	0.55*	0.78**	0.49	0.56*	0.2

Correlation coefficients (r_s) for differences between strains with respect to virus burden and immune response. (*$0.01 < p \leq 0.05$, **$0.001 < p \leq 0.01$, ***$p \leq 0.001$).

herpes virus (KHV) [46-48]. A cohabitation infection trial using the same HVI as the present study observed a relatively low level of infection in the gills, however few sampling points were used [34]. The currently presented viral load kinetics, backed up by the assays directly targeting cRNA and mRNA, suggest the presence of an early primary replication phase for the LVI in the gills, not observed for the HVI. Detection of both viruses in all fish at 6 h pi by seg8 real-time RT-PCR indicated rapid uptake of both viruses to the gills from the water. However, only the LVI amount displayed an early

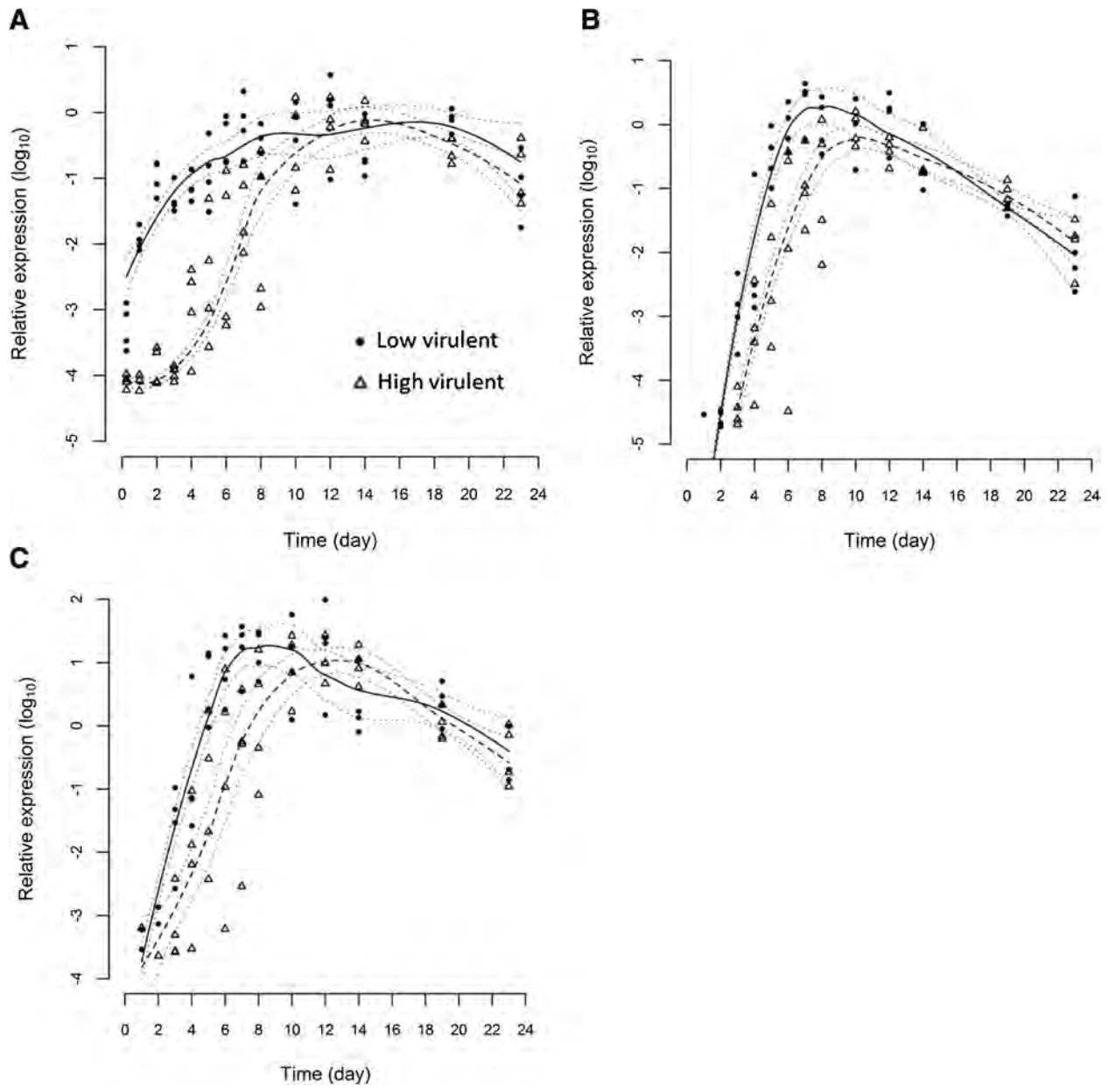


Figure 5 Statistical modelling of ISAV segment 7. Statistical modelling of viral segment 7 total RNA load profiles of high virulent (dashed line) and low virulent (solid line) strains measured by real-time RT-PCR from gill **(A)**, anterior kidney **(B)** and heart **(C)** tissues. Dotted lines indicate 95% confidence intervals.

immediate increase which can initially be attributed to the rapid production of mRNA in the time points that follow. The replicative intermediate, cRNA, was also detected in some fish from day 1. As expected this was at much lower levels throughout the experiment [38,44]. Therefore the production of cRNA had a negligible effect on the overall viral load compared to increasing mRNA production and accumulating genome (vRNA) from nascent virions. The subsequent effect of this early virus multiplication was clearly visible in the kidney and heart, where LVI was consistently detected in greater quantities each day up until day 8 pi. The early LVI replication (as indicated by the specific detection of the replicative intermediate cRNA) and accumulation of nascent virus particles in the gills, prior to dissemination, probably accounts for this. Interestingly, the putatively non-virulent ISAV HPR0 is primarily associated with detection in the gills only [9,49]. The gill results suggest HVI progresses more slowly into a generalised infection. However, the fact that HVI may utilise an

alternative entry point not examined in this study, cannot be excluded.

Virus virulence is a multifunctional trait and replication efficiency is one factor that has been associated with increased virulence. A study into the differential virulence mechanisms of two strains of IHNV of high or low virulence in salmonid fish was shown to be linked with faster in vivo kinetics and replication of the highly virulent virus [50,51]. Early innate host immune response was demonstrated to play a critical role and it was proposed that the lag in immune system stimulation might give the faster replicating highly virulent virus enough time to reach a threshold and outrun the host antiviral response [51]. In addition to the replication advantage, it was also established that the highly virulent IHNV had an advantage at the entry stage of the infection cycle, when data from immersion and IP-injection experiments were compared [52]. Contrary to this, investigations on simian immunodeficiency virus (SIV) have suggested high replication rates of the natural virus do not correspond with increased virulence [53]. In the present study following immersion challenge, whilst the LVI rapidly gained entry to gill cells and replicated immediately, it ultimately reached a lower peak viral load than the HVI and caused a lower mortality. We could argue this provides LVI with an evolutionary advantage over HVI. Replication to relatively high levels without killing all the available hosts may allow the virus to persist in a population and potentially spread further. However, further information on virus shedding rates is required to investigate this. Our data suggests that the LVI has an advantage at the entry stage in gills and also exhibited a higher replication rate during the early stages of infection. This finding agrees with previous experimental data demonstrating slower replication of a high pathogenic ISAV compared to a low pathogenic ISAV in Atlantic Salmon Kidney (ASK) cells at 20 °C [35]. The reason for apparent faster replication of LVI at early time points remains unknown, but could be due to enhanced viral RNA-dependant RNA polymerase (RdRp) activity. This activity could also be in conjunction with differential properties of the viral glycoproteins, such as more efficient particle attachment or membrane fusion, as demonstrated for highly pathogenic influenza A viruses [54,55]. At present, functional information on ISAV RdRp's, and on the links between RdRp's and surface proteins is limited.

The significant up-regulation of all four immune genes showed both LVI and HVI effectively stimulated the innate and adaptive immune systems. The response to both viruses was substantial and sustained. It was evident LVI disseminated more rapidly due to its replication at the earlier stages of infection, thus triggering an earlier systemic IFN-mediated immune response compared to that of HVI. Infection of TO cells with ISAV of low and high virulence suggested replication was a requirement for induction of immune genes [36]. The generation of dsRNA is a pivotal part of the replication process for many ssRNA viruses and longer stretches of dsRNA are key indicators of viral invasion to a cell [56]. In the present study, the detection of the replicative intermediary cRNA early in the gills signals its presence. Cells detect viral RNA and proteins via pathogen associated molecular pattern (PAMP) receptors, which in turn stimulate interferons and the antiviral response [32,57]. In this study, the more rapid systemic response induced by LVI might have provided a sufficient level of protection in a higher number of hosts, preventing this strain from reaching the damaging higher viral loads observed for HVI. In the latter half of the trial the immune genes of the HVI infected fish were up-regulated more in comparison to those of the LVI infected fish. Interferon responses to viral infection are usually transient and self-limited to avoid a prolonged anti-viral state which in itself can be detrimental to the host and interfere with haematopoiesis [57,58]. Indeed, the vast induction of cytokines and chemokines, generating a "cytokine storm" and overwhelming inflammatory responses, have been linked to highly pathogenic influenza virus pathogenesis [57,59,60]. The over-activation of IFNβ and tumour necrosis factor–α (TNFα) creates a powerful pro-inflammatory response compared to that of low pathogenic influenza viruses, tipping the balance of the response towards inflammation, contributing to tissue damage [61]. The possibility that the increased mortality caused by HVI, which coincided with high expression of immune markers was caused by similar immune mechanisms should not be excluded. This study only focussed on a very small aspect of the immune response, therefore a more in-depth analysis of a greater number of immune response genes in immersion challenged fish would be advantageous. What is clear from the present study, the immune response was sufficient to limit the infection by LVI while ineffectual at preventing HVI instigating a progressive infection causing an eventual fatal outcome.

RNA viruses have evolved diverse strategies to counteract and evade the host immune system. The influenza virus NS1 protein for example, is the primary antagonist of the innate immune response and remarkably effects many stages of the interferon response [62]. Two ISAV proteins, including the putative NS protein, have been linked to the antagonism of the IFN system [17,18], but it was not possible to relate these functions specifically here. In a study such as this, any viral IFN antagonistic effect within cells is likely to go unseen due to the vast cytokine inductions in neighbouring uninfected cells. Real-time PCR of segment 7 followed a similar profile to segment 8 in all three organs tested. However, correlation analysis suggested the segment 7 load, unlike segment 8,

is unlikely to be directly related to the immune gene expression in either the gill or the heart. This has two possible explanations. Firstly, the functions of the proteins encoded by these segments are very different and the expression may be time dependent. In addition, the quantities of the required proteins are also substantially different and neither segment assay differentiates the two ORFs. The M protein is the most abundant protein in the virion [14], thus more is required compared to the non- or minor- structural proteins encoded by segment 7. Comparing the segment 7 to segment 8 ratios of both viruses indicated the potentially interesting observation that HVI appears to generate less segment 7 RNA than LVI. The significance of this is unknown and remains an area of further investigation, although may partly explain the lack of correlation between segment 7 and immune genes.

In conclusion, this study indicated that low virulent ISAV (LVI) replicated earlier in the gills and disseminated throughout the host more efficiently following immersion challenge compared to the highly virulent virus (HVI). This suggests potential variation in tissue or even cell tropism. Rapid replication in gills stimulated a marked systemic immune response that may have provided some protection against LVI-induced pathogenesis, causing a limited infection. In contrast, the host response against HVI was less effectual, allowing the virus to reach a higher load causing a progressive infection and ultimately inducing significantly more mortality. Further work is required to elucidate the underlying factors that allow LVI to replicate more efficiently in the gills following immersion infection. Other factors such as variable HE activity allowing increased viral particle liberation to infect more cells, or differing abilities in immune system interference may have a role to play. In addition, because the HVI does not appear to access the gills as quickly, other entry points such as skin, eye, gut, should also be investigated. A greater understanding of the molecular and pathophysiological mechanisms of virulence and pathogenesis of LVI and HVI is critical in developing strategies and preventative measures to combat viral infection by ISAV.

Additional files

Additional file 1: Primers and probes used in RT-qPCR and RNA specific RT-qPCR. Table of details relating to all primer and probe sequences used in the standard real-time RT-PCR analysis and the RNA specific real-time RT-PCR analysis.

Additional file 2: Difference in expression of ISAV segment 7 compared to segment 8. Expression ratio of segment 7 compared to segment 8 in gill (A), anterior kidney (B) and heart (C) in fish infected with either low virulent (LVI) or highly virulent (HVI) ISAV.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AM carried out and supervised all molecular experimental work, analysed results and drafted the manuscript. YMH and AM carried out the RNA specific assays. MH and AM carried out statistical analysis. MH wrote the statistical sections in the manuscript. KF designed and organised the experimental challenge, prepared virus, and participated in the execution of the challenge and in data analysis. AM, MA, DHC, TM and IM participated in the design and execution of the experimental aquarium challenge and in data analysis. All authors contributed to the revision and editing of the manuscript and approved the final version.

Acknowledgments

The authors thank Mickael Fourrier and Rita Pettinello at Marine Scotland Science and Simon Weli at the Norwegian Veterinary Institute for laboratory assistance. Thanks also to the staff at the VESO Vikan research station, in particular our study director, Anne Berit Romstad. The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7, 2007–2013), Research Infrastructures action, under the grant agreement No. FP7-228394 (NADIR project), the Norwegian Research Council, HAVBRUK project No.186907 and 207024 and the Faroese research Council.

Author details

[1]Marine Scotland Science, Marine Laboratory, Aberdeen, Scotland, UK. [2]Norwegian Veterinary Institute, Oslo, Norway. [3]Food and Veterinary Authority, Tórshavn, Faroe Islands.

Received: 2 June 2014 Accepted: 24 July 2014
Published: 21 August 2014

References

1. Aamelfot M, Dale OB, Falk K: **Infectious salmon anaemia - pathogenesis and tropism.** *J Fish Dis* 2014, **37:**291–307.
2. Thorud K, Djupvik HO: **Infectious anaemia in Atlantic salmon (*Salmo salar* L.).** *Bull Eur Assoc Fish Pathol* 1988, **8:**109–111.
3. Bouchard D, Keleher W, Opitz HM, Blake S, Edwards KC, Nicholson BL: **Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada.** *Dis Aquat Organ* 1999, **35:**131–137.
4. Lovely JE, Dannevig BH, Falk K, Hutchin L, MacKinnon AM, Melville KJ, Rimstad E, Griffiths SG: **First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome.** *Dis Aquat Organ* 1999, **35:**145–148.
5. Mullins JE, Groman DB, Wadowska D: **Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada.** *Bull Eur Assoc Fish Pathol* 1998, **18:**110–114.
6. Murray AG, Munro LA, Wallace IS, Berx B, Pendrey DJ, Fraser D, Raynard RS: **Epidemiological investigation into the re-emergence and control of an outbreak of infectious salmon anaemia in the Shetland Islands, Scotland.** *Dis Aquat Organ* 2010, **91:**189–200.
7. Rodger HD, Turnbull T, Muir F, Millar S, Richards RH: **Infectious salmon anaemia (ISA) in the United Kingdom.** *Bull Eur Assoc Fish Pathol* 1998, **18:**115–116.
8. Anonymous: **ISA hits the Faroes.** *Fish Farming Int* 2000, **27:**47.
9. Christiansen DH, Østergaard PS, Snow M, Dale OB, Falk K: **A low-pathogenic variant of infectious salmon anemia virus (ISAV-HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (*Salmo salar* L.) in the Faroe Islands.** *J Gen Virol* 2011, **92:**909–918.
10. Godoy MG, Aedo A, Kibenge MJ, Groman DB, Yason CV, Grothusen H, Lisperguer A, Calbucura M, Avendano F, Imilan M, Jarpa M, Kibenge FS: **First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (*Salmo salar*) in Chile.** *BMC Vet Res* 2008, **4:**28.
11. Rimstad E, Dale OB, Dannevig BH, Falk K: **Infectious Salmon Anaemia**. In *Fish Diseases and Disorders, Volume 3: Viral, Bacterial and Fungal Infections.* Edited by Woo PTK, Bruno DW. Oxfordshire: CAB International; 2011:143–165.
12. Mjaaland S, Rimstad E, Falk K, Dannevig BH: **Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (*Salmo salar* L.): an orthomyxo-like virus in a teleost.** *J Virol* 1997, **71:**7681–7686.
13. Aspehaug V, Mikalsen AB, Snow M, Biering E, Villoing S: **Characterization of the infectious salmon anemia virus fusion protein.** *J Virol* 2005, **79:**12544–12553.

14. Falk K, Aspehaug V, Vlasak R, Endresen C: **Identification and characterization of viral structural proteins of infectious salmon anemia virus.** *J Virol* 2004, **78:**3063–3071.
15. Rimstad E, Mjaaland S, Snow M, Mikalsen AB, Cunningham CO: **Characterization of the infectious salmon anemia virus genomic segment that encodes the putative hemagglutinin.** *J Virol* 2001, **75:**5352–5356.
16. Biering E, Falk K, Hoel E, Thevarajan J, Joerink M, Nylund A, Endresen C, Krossøy B: **Segment 8 encodes a structural protein of infectious salmon anaemia virus (ISAV); the co-linear transcript from Segment 7 probably encodes a non-structural or minor structural protein.** *Dis Aquat Organ* 2002, **49:**117–122.
17. Garcia-Rosado E, Markussen T, Kileng O, Baekkevold ES, Robertsen B, Mjaaland S, Rimstad E: **Molecular and functional characterization of two infectious salmon anaemia virus (ISAV) proteins with type I interferon antagonizing activity.** *Virus Res* 2008, **133:**228–238.
18. McBeath AJA, Collet B, Paley R, Duraffour S, Aspehaug V, Biering E, Secombes CJ, Snow M: **Identification of an interferon antagonist protein encoded by segment 7 of infectious salmon anaemia virus.** *Virus Res* 2006, **115:**176–184.
19. Ramly RB, Olsen CM, Braaen S, Rimstad E: **Infectious salmon anaemia virus nuclear export protein is encoded by a spliced gene product of genomic segment 7.** *Virus Res* 2013, **177:**1–10.
20. Falk K, Namork E, Rimstad E, Mjaaland S, Dannevig BH: **Characterization of infectious salmon anemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar* L.).** *J Virol* 1997, **71:**9016–9023.
21. Snow M, McKay P, McBeath AJ, Black J, Doig F, Kerr R, Cunningham CO, Nylund A, Devold M: **Development, application and validation of a Taqman real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*).** *Dev Biol (Basel)* 2006, **126:**133–145.
22. Dannevig B, Falk K, Skjerve E: **Infectivity of internal tissues of Atlantic salmon, *Salmo salar* L, experimentally infected with the etiologic agent of infectious salmon anemia (ISA).** *J Fish Dis* 1994, **17:**613–622.
23. Hetland DL, Dale OB, Skjødt K, Press CM, Falk K: **Depletion of CD8 alpha cells from tissues of Atlantic salmon during the early stages of infection with high or low virulent strains of infectious salmon anaemia virus (ISAV).** *Dev Comp Immunol* 2011, **35:**817–826.
24. Kibenge FS, Kibenge MJ, Groman D, McGeachy S: ***In vivo* correlates of infectious salmon anemia virus pathogenesis in fish.** *J Gen Virol* 2006, **87:**2645–2652.
25. Mjaaland S, Markussen T, Sindre H, Kjøglum S, Dannevig BH, Larsen S, Grimholt U: **Susceptibility and immune responses following experimental infection of MHC compatible Atlantic salmon (*Salmo salar* L.) with different infectious salmon anaemia virus isolates.** *Arch Virol* 2005, **150:**2195–2216.
26. Ritchie RJ, McDonald JT, Glebe B, Young-Lai W, Johnsen E, Gagne N: **Comparative virulence of Infectious salmon anaemia virus isolates in Atlantic salmon, *Salmo salar* L.** *J Fish Dis* 2009, **32:**157–171.
27. Markussen T, Jonassen CM, Numanovic S, Braaen S, Hjortaas M, Nilsen H, Mjaaland S: **Evolutionary mechanisms involved in the virulence of infectious salmon anaemia virus (ISAV), a piscine orthomyxovirus.** *Virology* 2008, **374:**515–527.
28. Mjaaland S, Hungnes O, Teig A, Dannevig BH, Thorud K, Rimstad E: **Polymorphism in the infectious salmon anemia virus hemagglutinin gene: importance and possible implications for evolution and ecology of infectious salmon anemia disease.** *Virology* 2002, **304:**379–391.
29. Markussen T, Sindre H, Jonassen CM, Tengs T, Kristoffersen AB, Ramsell J, Numanovic S, Hjortaas MJ, Christiansen DH, Dale OB, Falk K: **Ultra-deep pyrosequencing of partial surface protein genes from infectious Salmon Anaemia Virus (ISAV) suggest novel mechanisms involved in transition to virulence.** *PLoS One* 2013, **8:**e81571.
30. Langevin C, Aleksejeva E, Passoni G, Palha N, Levraud JP, Boudinot P: **The antiviral innate immune response in fish: evolution and conservation of the IFN system.** *J Mol Biol* 2013, **425:**4904–4920.
31. Zou J, Secombes CJ: **Teleost fish interferons and their role in immunity.** *Dev Comp Immunol* 2011, **35:**1376–1387.
32. Collet B: **Innate immune responses of salmonid fish to viral infections.** *Dev Comp Immunol* 2014, **43:**160–173.
33. McBeath AJA, Snow M, Secombes CJ, Ellis AE, Collet B: **Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus.** *Fish Shellfish Immunol* 2007, **22:**230–241.
34. Jørgensen SM, Hetland DL, Press CM, Grimholt U, Gjøen T: **Effect of early infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes and type I and II interferon in Atlantic salmon (*Salmo salar* L.) tissues.** *Fish Shellfish Immunol* 2007, **23:**576–588.
35. Svingerud T, Holand JK, Robertsen B: **Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell culture.** *Virus Res* 2013, **177:**163–170.
36. Workenhe ST, Hori TS, Rise ML, Kibenge MJ, Kibenge FS: **Infectious salmon anaemia virus (ISAV) isolates induce distinct gene expression responses in the Atlantic salmon (*Salmo salar*) macrophage/dendritic-like cell line TO, assessed using genomic techniques.** *Mol Immunol* 2009, **46:**2955–2974.
37. McBeath A, Aamelfot M, Christiansen DH, Matejusova I, Markussen T, Kaldhusdal M, Dale OB, Weli SC, Falk K: **Immersion challenge with low and highly virulent infectious salmon anaemia virus reveals different pathogenesis in Atlantic salmon, *Salmo salar* L.** *J Fish Dis*. in press.
38. McBeath A, Bain N, Fourrier M, Collet B, Snow M: **A strand specific real-time RT-PCR method for the targeted detection of the three species (vRNA, cRNA and mRNA) of infectious salmon anaemia virus (ISAV) replicative RNA.** *J Virol Methods* 2013, **187:**65–71.
39. Zou J, Carrington A, Collet B, Dijkstra JM, Yoshiura Y, Bols N, Secombes C: **Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish.** *J Immunol* 2005, **175:**2484–2494.
40. R Core Team: *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing; [http://www.R-project.org/]. (Accessed 29 November 2012).
41. Cleveland WS: **Robust locally weighted regression and smoothing scatterpoints.** *J Am Stat Assoc* 1979, **74:**829–836.
42. Searle SR: *Linear Models.* New York: John Wiley and Sons; 1971.
43. Spearman C: **The proof and measurement of association between two things.** *Am J Psychol* 1904, **15:**72–101.
44. Hatada E, Hasegawa M, Mukaigawa J, Shimizu K, Fukuda R: **Control of influenza virus gene expression: quantitative analysis of each viral RNA species in infected cells.** *J Biochem* 1989, **105:**537–546.
45. Mikalsen AB, Teig A, Helleman AL, Mjaaland S, Rimstad E: **Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon *Salmo salar*.** *Dis Aquat Organ* 2001, **47:**175–181.
46. Costes B, Raj VS, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Bremont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin.** *J Virol* 2009, **83:**2819–2830.
47. Harmache A, LeBerre M, Droineau S, Giovannini M, Brémont M: **Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus.** *J Virol* 2006, **80:**3655–3659.
48. Quillet E, Dorson M, Aubard G, Torhy C: **In vitro assay to select rainbow trout with variable resistance/susceptibility to viral haemorrhagic septicaemia virus.** *Dis Aquat Organ* 2007, **76:**7–16.
49. McBeath AJ, Bain N, Snow M: **Surveillance for infectious salmon anaemia virus HPR0 in marine Atlantic salmon farms across Scotland.** *Dis Aquat Organ* 2009, **87:**161–169.
50. Penaranda MM, Purcell MK, Kurath G: **Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) include host entry and virus replication kinetics.** *J Gen Virol* 2009, **90:**2172–2182.
51. Purcell MK, Garver KA, Conway C, Elliott DG, Kurath G: **Infectious haematopoietic necrosis virus genogroup-specific virulence mechanisms in sockeye salmon, *Oncorhynchus nerka* (Walbaum), from Redfish Lake, Idaho.** *J Fish Dis* 2009, **32:**619–631.
52. Wargo AR, Kurath G: **In vivo fitness associated with high virulence in a vertebrate virus is a complex trait regulated by host entry, replication, and shedding.** *J Virol* 2011, **85:**3959–3967.
53. Pandrea I, Sodora DL, Silvestri G, Apetrei C: **Into the wild: simian immunodeficiency virus (SIV) infection in natural hosts.** *Trends Immunol* 2008, **29:**419–428.
54. Pappas C, Aguilar PV, Basler CF, Solorzano A, Zeng H, Perrone LA, Palese P, Garcia-Sastre A, Katz JM, Tumpey TM: **Single gene reassortants identify a critical role for PB1, HA, and NA in the high virulence of the 1918 pandemic influenza virus.** *Proc Natl Acad Sci U S A* 2008, **105:**3064–3069.

55. Grimm D, Staeheli P, Hufbauer M, Koerner I, Martinez-Sobrido L, Solorzano A, Garcia-Sastre A, Haller O, Kochs G: **Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene.** *Proc Natl Acad Sci U S A* 2007, **104:**6806–6811.
56. Wang Q, Carmichael GG: **Effects of length and location on the cellular response to double-stranded RNA.** *Microbiol Mol Biol Rev* 2004, **68:**432–452.
57. Kash JC: **Applications of high-throughput genomics to antiviral research: evasion of antiviral responses and activation of inflammation during fulminant RNA virus infection.** *Antiv Res* 2009, **83:**10–20.
58. Lin Q, Dong C, Cooper MD: **Impairment of T and B cell development by treatment with a type I interferon.** *J Exp Med* 1998, **187:**79–87.
59. Cilloniz C, Shinya K, Peng X, Korth MJ, Proll SC, Aicher LD, Carter VS, Chang JH, Kobasa D, Feldmann F, Strong JE, Feldmann H, Kawaoka Y, Katze MG: **Lethal influenza virus infection in macaques is associated with early dysregulation of inflammatory related genes.** *PLoS Pathog* 2009, **5:**e1000604.
60. Marsolais D, Hahm B, Walsh KB, Edelmann KH, McGavern D, Hatta Y, Kawaoka Y, Rosen H, Oldstone MB: **A critical role for the sphingosine analog AAL-R in dampening the cytokine response during influenza virus infection.** *Proc Natl Acad Sci U S A* 2009, **106:**1560–1565.
61. Lee SM, Gardy JL, Cheung CY, Cheung TK, Hui KP, Ip NY, Guan Y, Hancock RE, Peiris JS: **Systems-level comparison of host-responses elicited by avian H5N1 and seasonal H1N1 influenza viruses in primary human macrophages.** *PLoS One* 2009, **4:**e8072.
62. Wang BX, Fish EN: **The yin and yang of viruses and interferons.** *Trends Immunol* 2012, **33:**190–197.

doi:10.1186/s13567-014-0083-x
Cite this article as: McBeath *et al.*: Low virulent infectious salmon anaemia virus (ISAV) replicates and initiates the immune response earlier than a highly virulent virus in Atlantic salmon gills. *Veterinary Research* 2014 **45**:83.

EXHIBIT H-8

ICES WGPDMO REPORT 2016

SCICOM STEERING GROUP ON ECOSYSTEM PRESSURES AND IMPACTS

ICES CM 2016/SSGEPI:07

REF. SCICOM

Interim Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO)

17–20 February 2016

Virginia, USA

ICES International Council for the Exploration of the Sea

CIEM Conseil International pour l'Exploration de la Mer

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

Recommended format for purposes of citation:

ICES. 2016. Interim Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), 17–20 February 2016, Virginia, USA. ICES CM 2016/SSGEPI:07. 18 pp.

Contents

Executive summary

The ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) met on 17–20 February 2016 at the Virginia Institute of Marine Science, College of William & Mary, in Gloucester Point, Virginia USA. The meeting was chaired by Ryan Carnegie (USA) and attended by eight other participants representing eight ICES Member Countries.

The agenda included several topics related to diseases and pathology in wild and farmed fish and shellfish.

The group produced a report on new disease trends in wild and farmed fish and shellfish in the ICES area based on national reports from fifteen member countries. Notable reports for wild fish included first observations of salmon gill poxvirus in Canada and piscine reovirus in Denmark; the first observation of *Vibrio anguillarum* serotype O3 in Sweden; disease associated with oomycetes in Russia and Sweden; and prevalences of *Pseudoterranova decipiens* infection in the northern Baltic Sea that were unexpectedly low given the high prevalences in the southern Baltic. Reports for farmed fish included the first observation of piscine orthoreovirus in Ireland and range expansion of salmonid rickettsial septicaemia in western Canada. Additionally, wrasse and lumpfish cultured as cleaner fish for salmonid aquaculture were noted to be affected by bacterial diseases. Salmon louse control to minimize risk to wild fish poses an ongoing challenge to salmon aquaculture in the ICES region. In shellfish, observations for Pacific oysters included the association of *Vibrio aestuarianus* with significant mortality in Ireland, the detection of *Haplosporidium costale* in England representing a first record in Europe, and the detection of a *Marteilia* not identified to species in France. *Marteilia cochillia* is an important emerging concern, causing significant disease and mortality in cockles in Spain.

Work on four additional documents was discussed, including a summary of the role of *Vibrio* pathogens contributing to mortalities in shellfish aquaculture, a synthesis on the contemporary status of oyster pathogen *Bonamia ostreae*, and a description of the distribution of amoebic gill disease in marine salmon farms, all to be prepared for publication in scientific journals; and a compilation of pathogen screening in wild salmonids, to be presented in the final WGPDMO report. The Fish Disease Index package in R was described with plans to simplify the interface between a MS Excel input spreadsheet and the R program. The package now includes features to define new FDI, which will allow the index to be applied to species other than those for which it was developed. The FDI will be circulated for testing in 2016.

One new and two revised ICES Identification Leaflets for Diseases and Parasites of Fish and Shellfish were published, including 'Francisellosis of Atlantic cod', '*Mytilicola intestinalis*' parasitism, and 'Furunculosis'. Two additional leaflets have been submitted, fourteen new leaflets have been proposed, and the entire remaining catalog has been reviewed to initiate revisions of leaflets not already updated.

The work plan for the cycle has been revised to include a new ToR i on the generation of a standard reporting template to improve data collection concerning sample sizes and pathogen prevalences and allow improved resolution of disease trends.

1 Administrative details

Working Group name

Working Group on Pathology and Diseases of Marine Organisms (WGPDMO)

Year of Appointment

2016

Reporting year within current cycle (1, 2 or 3)

1

Chair(s)

Ryan B. Carnegie, USA

Meeting venue

Gloucester Point, Virginia, USA

Meeting dates

17–20 February 2016

2 Terms of Reference a) – z)

ToR a) Summarize new and emerging disease trends in wild and cultured fish, molluscs and crustaceans based on national reports.

ToR b) Deliver leaflets on pathology and diseases of marine organisms.

ToR c) Synthesize information on the spread and impact of *Bonamia ostreae* in flat oysters in the ICES area.

ToR d) Summarise the role of *Vibrio* sp. pathogens contributing to mortalities in shellfish aquaculture.

ToR e) Prepare a report describing the occurrence and spread of amoebic gill disease (AGD) in marine salmonid farming in the ICES area.

ToR f) Compile information on pathogen screening of wild salmonids in the ICES member states.

ToR g) Evaluate applicability of the Fish Disease Index (FDI) by using the R package following newly developed guidelines.

ToR h) Provide expert knowledge and management advice on fish and shellfish diseases, if requested, and related data to the ICES Data Centre.

3 Summary of Work plan

ToR a) New disease conditions and trends in diseases of wild and cultured marine organisms will be reviewed. This is an annual, ongoing ToR for WGPDMO and will provide information for ToRs c-f.

ToR b) A number of ICES publications currently in preparation will be reviewed by WGPDMO. This is an ongoing, annual ToR.

ToR c) *Bonamia ostreae* is a major pathogen of European flat oysters that has expanded its range in recent years. The present distributional status, recent trends in parasite activity, and the effectiveness of contemporary management strategies will be summarized, with perspective included on related species *Bonamia exitiosa*, recently documented in European systems.

ToR d) *Vibrio* bacteria have long been associated with larval production problems in shellfish hatcheries, but the potential impacts of vibriosis in sub-market and market-sized Pacific oysters in European production areas has become an important emerging concern. This ToR will summarize the available science to provide a synthesis on current knowledge on *Vibrio* impacts and highlight critical gaps in our understanding of these species.

ToR e) AGD has emerged as a significant issue for salmon farming in the Atlantic. This ToR will produce a report describing the spread and impact of this disease and current measures being used to mitigate its effects. It will identify knowledge gaps and future areas for research.

ToR f) Many ICES member countries screen wild broodstock used for restocking purposes for disease pathogens. This ToR will produce a report compiling information on diseases and methods used in order to prepare a common approach to screening and assess the effectiveness of current practices.

ToR g) This ToR will produce an assessment of the applicability based on its trial use by participants from among the group.

ToR h) This is an annual ToR in compliance with a requests from the ICES Data Centre.

4 List of Outcomes and Achievements of the WG in this delivery period

- A report on new disease trends in wild and farmed fish and shellfish in ICES Member Countries, which is the only annual expert report available on this topic
- Publication of ICES Disease Leaflet No. 24: *Mytilicola intestinalis* parasitism (Bignell, revised leaflet), No. 37: Furunculosis (Bruno, revised leaflet) and No. 64: Francisellosis of Atlantic cod (Alfjordan and Ruane, new leaflet)
- Submission of a new ICES Disease Leaflet on Brown ring disease in clams (Paillard) as well as a revision of No. 42: *Exophiala* (Bruno)

5 Progress report on ToRs and workplan

5.1 Summarize new and emerging disease trends in wild and cultured fish, molluscs and crustaceans based on national reports (ToR a)

The update in the following sections is based on national reports for 2015 submitted by Canada, Denmark, England & Wales, Finland, France, Germany, Ireland, the Netherlands, Norway, Poland, Russia, Scotland, Spain, Sweden and the USA. It documents significant observations and highlights the major trends in newly emerging diseases and in those identified as being important in previous years.

5.1.1 Wild Fish

Viruses

Salmon gill poxvirus (SGPV) – Reported for the first time in Canada from a healthy adult Atlantic salmon in the Magaguadavic River, New Brunswick. The finding was based on cytopathology and high-throughput DNA sequencing.

Piscine reovirus (PRV) – Reported from Denmark in 2014 for the first time, 6% of 176 Atlantic salmon brood-stock tested positive by qPCR. The virus was later detected in progeny (fry) from the affected brood fish despite disinfection of eggs. Eight wild brown trout were found to be negative for the virus.

Infectious pancreatic necrosis virus (IPNV) – In mid-Norway, the virus was detected in gill samples in 7 of 670 of returning Atlantic salmon in four rivers in 2013 and 2014.

Infectious salmon anaemia virus (ISAV) – In mid-Norway, the virus (HPR0) was detected in gill samples in 16 of 670 of returning Atlantic salmon in four rivers in 2013 and 2014, and in 2014, the virus was also detected in 5 of 204 Atlantic salmon and 2 of 18 sea trout caught in marine estuaries in the same region.

Viral haemorrhagic septicaemia virus (VHSV) – A rare observation of Genotype 1b was made in a Baltic cod from Hanö Bay, Sweden (ICES district SD 25). The fish also showed signs of fin rot, purulent exudate, splenic granulomas, endo- and pericarditis, anaemia and peritoneal haemorrhage.

Bacteria

***Vibrio anguillarum* –** In Sweden, mass mortality among Atlantic herring from the island of Orust (ICES district 21) was associated with serotype O3 ("Pacific herring serotype"), not previously reported from Sweden.

Acute/healing skin ulcerations (U) – In Baltic cod, the prevalence was 3% in waters of Poland (n=17,748), Russia (n=945) and Sweden (n=3,940). For Polish waters, this was the lowest prevalence observed since 2010. For European flounder in Swedish waters, prevalence was 1% (n=4,895).

Fungi

***Paranucleospora theridion* –** In mid-Norway, prevalence ranged from 7% to 70% during testing of nearly 900 returning Atlantic salmon between 2013 and 2014.

Oomycetes

Saprolegnia – In July–October 2015, in the Kola River (Barents Sea basin of Russia), infection in adult Atlantic salmon, European whitefish and minnow was observed for the first time with associated lethargy and mortality. Similar observations, reported in 2014 from Finland and Sweden, persisted in 2015 in Sweden. A diagnosis of ulcerative dermal necrosis (UDN) was made from a sample of 5 fish not showing oomycete growth.

Parasites

Protists

***Sphaerothecum*-like parasite (Mesomycetozoa)** – Prevalence in common dab from the North Sea stations West Dogger Bank and North Dogger Bank was 3% (n=80), and at Indefatigable Bank was 1% (n=80). Infections were observed in the liver and kidney at low intensity, usually associated with a granulomatous host response.

Ichthyobodo salmonis – In mid-Norway, prevalence was approximately 50% during testing of nearly 900 returning Atlantic salmon between 2013 and 2014.

Myxozoa

Parvicapsula pseudobranchicola – In mid-Norway, prevalence was approximately 38% during testing of nearly 900 returning Atlantic salmon between 2013 and 2014.

Nematoda

Contracaecum osculatum – The increasing trend reported earlier from Baltic cod continued in 2015 with data from Denmark, Poland, Russia and Sweden. Small cod (35–40 cm) harboured intense infections of up to 300 parasites per fish (n=66 liver samples).

Pseudoterranova decipiens – In the Barents Sea (ICES areas 1 and 2b), infections in long-rough dab increased between 2014 and 2015 from 21% to 30% and 12% to 35%, respectively. In Atlantic cod from area 2b, prevalence increased from 17% to 24%. In Baltic cod caught east of Bornholm, prevalence up to 55% and intensity up to 56 worms per fish were reported. In Swedish waters, over 60% of Baltic cod and 100% of shorthorn sculpin were infected, indicating that the upward trend reported earlier continues. Further north into the Baltic, around Gotland, prevalence in cod was 7%, and in shorthorn sculpin was 9%. Finally in the archipelago of Stockholm and the sea of Åland, prevalence decreased to nearly zero, with only a single nematode found in over 200 examined fish. It is not known why the prevalence was so low in northern areas despite the presence of significant concentrations of grey seal. Another factor such as salinity or the absence of suitable intermediate hosts may be responsible.

Anisakis simplex – Prevalence in common dab from waters of England and Wales remains high (13–55%) at the majority of fishing stations in the North Sea. The Tyne Tees region exhibited the highest prevalences, of 37%, 55% and 20% at Amble, Flamborough and Tees Bay, respectively. In ICES area 1, prevalence in polar cod decreased from 64% to 33% and in capelin from area 2b, from 52% to 28%. Prevalence in cod from area 2b increased from 93% to 100% and in herring from area 1, there was an increase from 47% to 64%. In Polish waters, prevalence in cod decreased from 31% (n=278) to 15% (n=303). The prevalence of infection was higher in the Gulf of Gdansk (30%) than in the Western Baltic

(9%) and middle coast (5%). Previously, higher values were recorded in the western and middle Baltic. For Baltic herring in Polish waters, analysis of prevalence data using a generalized linear model (GLM) revealed that estimated year effects for 2013, 2014 and 2015 strongly decreased in comparison to effects of 2011 and 2012. A similar decrease was revealed for intensity.

Monogenea

***Gyrodactylus salaris* –** The parasite was discovered for the first time in the River Rolfsån on the Swedish west coast. Together with the nearby River Kungsbackaån, this was one of two water systems previously recognised as *G. salaris*–free.

Other diseases

Eye Pathology – Persisting in capelin (3%), polar cod (2%) and Atlantic cod (0.4%) from the Barents Sea as acute exophthalmos, cataract, red eyes, or ocular degeneration.

Hyperpigmentation – Continued to be observed in common dab at relatively high prevalence in the North Sea in 2015 compared to the Irish Sea, Severn and English Channel regions (between 0% and 25% in 2014): Amble (30%), Tees Bay (15%), Flamborough (43%), North Dogger (35%), Central Dogger (49%), West Dogger (49%), Indefatigable Bank (39%), and Off Humber (39%). Moreover, North Sea regions continued to show an increasing trend concerning prevalence of this condition.

Effects of munition dumpsites – Based on German data, no major differences in the health status of Baltic cod have been observed between munition dumpsites and reference areas in the western and eastern Baltic Sea. However, condition factors of cod in the main dumpsite east of Bornholm were significantly lower compared to all other study areas.

Conclusions

- A salmon gill poxvirus (SGPV) sequence was obtained from a healthy Atlantic salmon in New Brunswick, eastern Canada.
- Piscine reovirus in Atlantic salmon was reported for the first time from Denmark.
- In Sweden, mass mortality among Atlantic herring from the island of Orust was associated with *Vibrio anguillarum* serotype O3.
- High morbidity and mortality among migrating Atlantic salmon in Russia (Kola River) and Sweden (Baltic Sea, west coast) were associated with oomycete infection. UDN was diagnosed from samples not showing oomycete growth.
- Prevalence of *Pseudoterranova decipiens* in Baltic cod and shorthorn sculpin declined along a salinity gradient in the Baltic Sea despite the presence of large seal populations.
- No measurable differences in health status of Baltic cod were attributed to proximity to munition dumpsites. A reduction in condition factor among cod collected near dumpsites was measured.

5.1.2 Farmed Fish

Viruses

Infectious pancreatic necrosis virus (IPNV) – In Sweden, two cases of IPN serotype ab were diagnosed in rainbow trout in a national screening program. One of the farms was in the Baltic Sea, the other in an inland lake. In Norway, the number of cases declined from 48 in 2014 to 30 in 2015, continuing a trend reported previously. IPNV was found in Atlantic halibut fry in Norway on two occasions.

Infectious salmon anaemia virus (ISAV) – The disease was diagnosed in 15 Atlantic salmon farms in Norway, an increase from 10 farms in each of the two previous years. Only three cases were considered primary outbreaks, one in brood fish, one at a sea site, and the third in a smolt farm. Four secondary cases received fish from the smolt farm. The remaining secondary cases were likely caused by horizontal spread from neighboring farms. Two epidemics in northern Norway from 2013 and 2014 are still not declared eradicated. At two sites, rainbow trout were infected following infection of Atlantic salmon at the same site. These cases are the first registered in rainbow trout under ordinary farming conditions. In eastern Canada, sporadic outbreaks with the North American genotype persist, however surveillance revealed a high prevalence of European type HPR0 strains. In western Canada, 0 of 2207 Atlantic salmon tested positive by qRT-PCR.

Salmonid alphavirus (SAV) – In Norway, there are two endemic regions with two subtypes of the virus, SAV2 and SAV3, and the northernmost part of the country is surveilled to maintain SAV-free status. One case of SAV2 was seen in Atlantic salmon in this region, and the affected population was immediately culled. During 2014 and 2015, there have been cases of pancreas disease (PD) caused by SAV2 in the SAV3-zone. The number of PD cases in 2015 was 135, close to the historically high number of 142 in 2014. Ireland experienced seven outbreaks of PD, after only three in 2014.

Piscine orthoreovirus (PRV) – Heart and skeletal muscle inflammation (HSMI) was diagnosed for the first time in Ireland at one marine Atlantic salmon site, and detection of PRV was confirmed by qPCR. Mortality was reported to be low. In Norway, the number of HSMI outbreaks in Atlantic salmon was 135, a reduction from the historical peak of 181 in 2014 that coincided with national delisting of this disease. Using qRT-PCR, the virus was detected for the first time in eastern Canada in all Atlantic salmon from one lot held in quarantine. In addition, 6 of 11 salmon originating from another hatchery and held at a government research facility tested positive. None of the Canadian salmon were examined histologically for evidence of HSMI.

***Onchorhynchus mykiss* reovirus –** A new viral disease in rainbow trout first reported in 2013 from four different hatcheries in Norway was documented in the WGPDMO report from 2015. This disease had also caused mortality in fish transferred to seawater. Sequencing of the new viral agent showed that it is related to PRV in Atlantic salmon. No disease outbreaks have been registered in 2015, however, the virus was detected at 9 marine sites from among 50 farms tested.

Salmon gill poxvirus (SGPV) – Salmon gill poxvirus disease has been known in Norway since 1995. The first genome sequence of this DNA-virus was described in 2015. SGPV was diagnosed in a total of 18 Atlantic salmon farms last year, 15 marine sites and three smolt farms.

Bacteria

***Aeromonas salmonicida* –** One case was diagnosed in a marine Atlantic salmon site in Ireland. In Norway, *A. salmonicida* subsp. *salmonicida* was isolated in one case of increased mortalities in lumpfish transferred to a sea-site containing vaccinated Atlantic salmon, which were not affected. In Scotland, atypical *A. salmonicida* has been detected in moribund ballan wrasse being used as cleaner fish for farmed Atlantic salmon. In Norway, atypical *A. salmonicida* has been diagnosed in lumpfish used as cleaner fish in 51 cases, and in wrasses in 32 cases.

Atypical *A. salmonicida* was found in three cases in Norway, all involving Atlantic halibut fry.

Yersinia ruckeri – Norway had 34 cases of yersiniosis in Atlantic salmon in 2015, eight in smolt farms, 25 in sea farms, and one in brood fish. Detected cases have increased over the last four to five years. As a consequence, smolt farms are increasingly using vaccines.

***Moritella viscosa*/winter ulcers –** Winter ulcer syndrome was diagnosed in Atlantic salmon from three sites in Scotland and three sites in Ireland. In Norway, 57 cases of winter ulcers in Atlantic salmon and four cases in rainbow trout were diagnosed, compared to 44 cases in salmonids in 2014.

***Vibrio-infections* –** In Norway, *Vibrio anguillarum* has been isolated from diseased cleaner fish used to control salmon lice. The bacterium was detected in lumpfish from twelve farms and wrasses from two. Three cases of *V. ordalii* have been reported in lumpfish.

Flavobacterium*/*Flexibacter – Three cases of infection with *Flavobacterium psychrophilum* in rainbow trout were reported in Norway, two from marine sites and the third from an inland farm. Two cases were registered in 2014, and septicemic flavobacteriosis in rainbow trout has been a list 3 disease in Norway since that time.

***Pasteurella*/*Pseudomonas* –** In lumpfish from Norway, *Pasteurella* sp. was isolated in 14 cases and *Pseudomonas anguilliseptica* in four.

***Piscirickettsia salmonis* –** The range of salmonid rickettsial septicaemia in Atlantic salmon in western Canada expanded to a new management zone and the disease now occurs throughout the year in some locations. Between 2013 and 2015, the annual number of diagnoses in Atlantic salmon has increased from 8.5% to 29% and in Pacific salmon, from 4% to 38%.

Parasites

Crustacea

***Lepeophtheirus salmonis* –** Salmon lice control remains the most important challenge with regard to Atlantic salmon culture in ICES member countries.

Paramoebida

***Paramoeba perurans* –** In Canada, infection has been reported in two new management zones along with increased mortality in zones that had earlier been identified as affected. AGD prevalence in Norway and Scotland has stabilized after increasing in previous years.

Conclusions

- Salmon louse control for the purposes of minimizing risk to wild fish continues to pose a great challenge to salmon aquaculture.
- Amoebic gill disease (AGD) prevalence has increased in Canada and stabilized elsewhere.
- Wrasse and lumpfish cultured as cleaner fish for use in salmon aquaculture have been recognized as affected by bacterial diseases.
- Spatial and temporal ranges of salmonid rickettsial septicaemia caused by *Piscirickettsia salmonis* in Atlantic salmon have expanded in western Canada, coincident with an increase in the number of diagnoses.

5.1.3 Wild and farmed molluscs and crustaceans

Viruses

Oyster Herpes Virus – OsHV-1 µVar continues to be detected in association with mortality in Pacific oysters in France. *Vibrio aestuarianus* detection by PCR is often observed in these cases.

Low levels of OsHV-1 DNA have been observed in mortality cases involving a number of bivalve species in France, including Mediterranean mussels (1 of 1 case) and cockles (1 of 4 cases), with accompanying *Vibrio splendidus* DNA detection in both cases. OsHV-1 DNA was also detected in Manila clams along with *Perkinsus* sp. in 1 of 2 cases with 40–50 % mortality.

Western Canada remains OsHV-1-free based on qPCR analysis of 40 seed oyster samples. OSHV-1 µVar-related mortalities in Pacific oysters have ceased in Norway and Sweden following outbreaks in 2014, although the infection status of populations there was not determined.

In southern England the virus has spread within areas already affected, from the Blackwater to the Mersea Creeks and the Colne, and from the River Crouch to the River Roach in Essex It has also spread, to new parts of the coast, along the north Kent coast in feral populations at Minnis Bay and Pegwell Bay as well as to farmed juvenile Pacific oysters in the River Teign in South Devon. The virus was detected in a new area in Ireland at low prevalence (2 of 30 Pacific oysters) and with no mortality. The virus is now considered to be present in 34 out of 43 Pacific oyster growing areas in that country.

OsHV-1 was detected in Pacific oysters from Bodega Bay, California, USA, following very high summer mortalities (~75% in the affected groups) associated with elevated water temperatures. This represents a new geographic record, the pathogen previously having been detected only in Tomales Bay, California. The viral genotype has not been determined.

Bacteria

***Vibrio aestuarianus* –** Continues to be detected in Pacific oysters and cockles from France. In Pacific oysters it was detected by qPCR in association with mortality in all age classes. In cockles, mortalities of 32–50% were observed and *V. aestuarianus* DNA was

detected in three of four of these cases. Vibrios belonging to the *V. splendidus* group were also detected in two of the batches of cockles that were positive for *V. aestuarianus*.

Significant mortality events affecting principally adult Pacific oysters occurred in 16 bays around the coast of Ireland. Juveniles were affected in seven of 16 bays and spat mortality was recorded in four of 16 bays. *V. aestuarianus* was identified at high levels in fourteen of the bays by qPCR. The bacterium was also cultured from these same bays and pathology consistent with infection (as described by the EURL) was evident in the majority of sites. Mortality levels varied widely (10–90%), and environmental influences such as prolonged heavy rains and a bloom of alga *Karenia mikimotoi* may have contributed to mortality in places.

***Vibrio* spp. –** Vibrios belonging to the *V. splendidus* group were also detected in Mediterranean mussels in France (14 of 15 positive batches) in association with abnormal mortality ranging from 10 to 50%. Similar events occurred in 2014. A number of other Vibrios including *Vibrio ostreicida* , *Vibrio tubiashii* subsp. *europeus* and *Vibrio bivalvicida* have been reported as contributing to problems affecting production of larval and spat stages of bivalve molluscs in Spain, including carpet shell, Manila, pullet and wedge clams, and arched, grooved and pod razor clams.

Candidatus *Xenohaliotis californiensis* – The northward distributional extent of *X. californiensis* in red abalone is now identified as Bodega Bay, California, where the pathogen was first observed in 2010. A phage infecting *X. californiensis* appears to have been present in this red abalone population in 2015 after having not been detected in 2010. Phage infection of *X. californiensis* in black abalone has suggested reduced bacterial pathogenicity.

Parasites

***Marteilia refringens* –** Found in Mediterranean mussels in France in 3 of 15 batches where mortalities were seen. The same three batches were positive for Vibrios from the *V. splendidus* group. In Sweden *M. refringens* continues to be present albeit at lower prevalence (3%) in blue mussels but without observation in flat oysters.

***Marteilia cochillia* –** Reported as causing significant disease and mortality in cockle populations in Spain.

***Marteilia* sp. –** Was detected histologically in adult Pacific oysters in France at low prevalence and with no observed mortalities. Three of four sampled batches were positive, with detection in 1 of 39, 1 of 40 and 5 of 40 oysters, respectively.

***Haplosporidium costale* –** Detected following a mortality event in farmed Pacific oysters from the River Dart estuary at the end of 2015. Histological analysis revealed low-intensity infections in two of 30 juvenile oysters that were confirmed to be *H. costale* through DNA sequence analysis. The role of the parasite in the observed oyster mortality is uncertain. This is the first reported case in Europe.

***Haplosporidium nelsoni* –** A sporulating haplosporidian infection observed in one of 30 Pacific oysters undergoing mortality in a site in the southwest of Ireland in 2013 has been confirmed using DNA sequence analysis to be *H. nelsoni*. In the Virginia portion of Chesapeake Bay, USA, unexpectedly high prevalences and intensities were observed in aquaculture industry samples of Eastern oysters evaluated in spring and summer, with

maximum prevalence reaching 40% and serious infections common. Typical prevalence does not exceed 10%. Distribution of the parasite was found to have expanded in the Maryland portion of Chesapeake Bay as well.

Hematodinium perezi – Prevalence in juvenile blue crabs from the Eastern Shore of Virginia, USA, has been found to be 100% in multiple annual samples over 2012–2015, far higher than prevalence in adults, which is typically about 30% in non-epizootic years. While noted earlier in a more limited study, this observation brings a renewed focus to the role of juvenile crabs in the epidemiology of this pathogen.

Conclusions

- The distribution of OsHV-1 in California, USA, has expanded to Bodega Bay.
- *Vibrio aestuarianus* was associated with significant mortality in Pacific oysters in Ireland.
- *Marteilia cochillia* has a significant impact on cockle populations in Spain.
- *Marteilia* sp. was detected by histology at low levels in adult Pacific oysters in France.
- *Haplosporidium costale* was detected for the first time in Europe, in a case from southwest England.
- *Haplosporidium nelsoni* has been detected at increasing prevalences and intensities on the East Coast of the USA.

5.2 Deliver leaflets on pathology and diseases of marine organisms (ToR b)

At the 2015 WGPDMO meeting it was agreed to update all disease leaflets during the next 3 years and also to increase the visibility and relevance of the leaflets.

Since the 2015 meeting the following new leaflet has been published.

- No. 64: Francisellosis of Atlantic cod (Alfjordan and Ruane)

In addition two revised leaflets have also been published.

- No. 24: *Mytilicola intestinalis* parasitism (Bignell)
- No. 37: Furunculosis (Bruno)

Further leaflets have been submitted and will be published in the near future.

- Brown ring disease in clams (Paillard) (new leaflet)
- No. 42: *Exophiala* (Bruno) (revised leaflet)

It remains important for the WGPDMO to continue to propose titles of new leaflets and to suggest potential authors for these so that the series remains current with up to date and relevant information. In addition, the editor has contacted authors for production of further revised leaflets which are to be submitted during 2016. As part of the ongoing task to update all remaining leaflets more than ten years old the WGPDMO reviewed the list of published leaflets and identified members to either take responsibility to produce a revised leaflet or to propose an alternative author to the editor.

As part of the ToR on updates on 'new disease trends' the group proposed the following emerging disease conditions that should generate new disease leaflets:

1) *Mikrocytos* spp. (Carnegie)
2) *Bonamia exitiosa* (Carnegie)
3) Ostreid herpesvirus (Renault)
4) Infectious salmon anaemia (ISA) (Falk)
5) Pancreas disease (PD) (Taksdal)
6) *Haematodinium* (Stentiford)
7) X-cell in dab (Feist and Bass)
8) Vibriosis in oysters (Renault)
9) Gonadal neoplasia in bivalves (Renault)
10) Tenacibaculosis in farmed fish (Jones)
11) Vibriosis in farmed salmonids (Lillehaug)
12) *Sphaerothecum* in dab (Feist and Paley)
13) Mycobacteriosis in wild fish (Madsen)
14) QPX in hard clams (Smolowitz)

5.3 Synthesize information on the spread and impact of *Bonamia ostreae* in flat oysters in the ICES area (ToR c)

Bonamiosis is a disease notifiable both to the OIE and the EU (under Directive 2006/88/EC) which has recently spread to new areas. In 2014 *B. ostreae* was detected in the Limfjorden in Denmark, representing the first detection of the parasite in native flat oysters in the area. In 2008 *B. ostreae* was found in Norway, until now the only time that it was found in this specific area. In the UK there has also been a range expansion recently, while the range in Ireland has been stable for a decade. In the USA the parasite remains endemic in flat oyster populations on Atlantic and Pacific Coasts, although with minimal impacts on those host populations in recent years. Over the next two years the WGPDMO will prepare a report synthesizing current knowledge on the distribution of *B. ostreae* and its impacts on flat oyster aquaculture and fisheries in ICES member countries. Perspective will be included on the related species *Bonamia exitiosa*, considered an emerging pathogen in European systems, and on the effectiveness of management strategies for control of both these pathogens.

5.4 Summarise the role of *Vibrio* sp. pathogens contributing to mortalities in shellfish aquaculture (ToR d)

Vibrio bacteria pathogenic to Pacific oysters and other bivalve molluscs have been increasingly documented in WGPDMO national reports from the last several years, with 2015 no exception. Whilst it is becoming increasingly apparent that particular species such as *V. aestuarianus* and *V. splendidus* are involved in mortalities observed in aquacultured bivalves in natural waters, additional species have been reported as pathogens in hatchery and nursery environments, for example in 2015 in Spain. Still, there exists a lack of clarity in relation to the pathogenic role of different vibrios, particularly where multiple species or other pathogens such as the OsHV-1 µVar are detected in a single event. Over the next two years the WGPDMO will provide a synthesis on the current state of knowledge relating to these pathogens through a review of the existing literature and

data from events which have occurred in recent years, with a view toward identifying key knowledge gaps to be addressed through future research. The group's objective will be to elucidate the established roles of different *Vibrio* species in mortalities in both wild and aquaculture populations. Concurrently, a new EU project "Vivaldi" (*Preventing and mitigating farmed bivalve diseases*) includes among its aims an examination of the roles of the pathogens OsHV-1 and *Vibrio* species and their interactions. Relevant Vivaldi participants will be included as collaborators in the review being undertaken by the WGPDMO to ensure that the review is informed by results from the Vivaldi project.

5.5 Prepare a report describing the occurrence and spread of amoebic gill disease (AGD) in marine salmonid farming in the ICES area (ToR e)

AGD has emerged as a significant issue for salmon farming in ICES member countries. Over the next two years, the WGPDMO will prepare a report describing the spread, impact and current measures taken to mitigate effects of the disease and identify knowledge gaps and future areas for research. A recent paper (Oldham *et al.* 2016) provides an epidemiological review of the incidence and distribution of AGD. Our report will utilize recent information, including data from WGPDMO national reports.

5.6 Compile information on pathogen screening of wild salmonids in the ICES member states (ToR f)

Many ICES member countries screen wild broodstock used for restocking purposes for disease pathogens. Over the next two years, the WGPDMO will compile information on diseases and methods used in these screening efforts. The goal of the report is to describe the screening methods used and their effectiveness and to determine the practicalities of adopting a common approach to screening.

5.7 Evaluate applicability of the Fish Disease Index by using the R package following newly developed guidelines (ToR g)

The present version of the R package is able to perform following actions: read rawfish disease data (ICES format or user-supplied), calculate descriptive statistics, calculate disease prevalence including confidence limits (per area, over time), calculate FDI values (raw and standardized, per individual and per population), do assessment of FDIs based on BAC and EAC in a traffic light fashion, display the assessment on a map, do a long-term trend assessment. Standard versions of the FDI for common dab (*Limanda limanda*), cod (*Gadus morhua*) and flounder (*Platichthys flesus*), are included in the package. The package also contains features to define new FDI and to derive new BAC and EAC. User input to the programme is done via an Excel spreadsheet into which all necessary inputs are entered. At present, this spreadsheet has to be stored and subsequently the user has to start the R programme manually. This procedure will be simplified by allowing to start the programme directly from the Excel interface. This change will be done in the next weeks. The programme will subsequently be circulated to volunteering WGPDMO members for testing.

5.8 Provide expert knowledge and management advice on fish and shellfish diseases, if requested, and related data to the ICES Data Centre (ToR h)

Members of the WGPDMO continue to provide support to the ICES Data Centre in relation to the clarification of details concerning the submission of data.

6 Revisions to the work plan and justification

Proposed new ToR i: Development of a standard template for National Reports (D. Cheslett, S. Jones, W. Wosniok).

Justification: Variability exists within the National Reports for wild and farmed finfish and shellfish regarding disease occurrence and how it is reported. More comprehensive and uniform presentation of pathogen prevalences and sample sizes in particular over time will allow improved resolution of increasing or decreasing trends in disease activity reported annually in ToR a.

7 Next meetings

The 2017 meeting of the WGPDMO will take place at the National Marine Fisheries Research Institute, Gdynia, Poland, 14–18 February 2017.

The location and dates of the 2018 meeting of the WGPDMO and final meeting of this reporting cycle remain to be determined.

Annex 1: List of participants

Name	Address	Phone/Fax	Email
Marlene Areskog	National Veterinary Institute, 751 89 Uppsala, Sweden	+4618-674076, +4670-7244076	marlene.areskog@sva.se
Ryan B. Carnegie (Chair)	Virginia Institute of Marine Science P.O. Box 934 Gloucester Point, Virginia 23062, USA	1-804-684-7713	carnegie@vims.edu
Deborah Cheslett	Marine Institute, Rinville, Oranmore, Co. Galway, Ireland	+353 91 387249	deborah.cheslett@marine.ie
Stephen W. Feist	Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset. DT4 8UB. UK	+44 (1305) 206662	stephen.feist@cefas.co.uk
Susan E. Ford	Rutgers University Haskin Shellfish Research Laboratory 6959 Miller Avenue Port Norris, NJ 08349, USA	1-757-645-3163	susan@hsrl.rutgers.edu
Simon R. M. Jones	Fisheries and Oceans Canada Pacific Biological Station 3190 Hammond bay Road Nanaimo, BC, V9T 6N7 Canada	1-250-729-8351	simon.jones@dfo-mpo.gc.ca
Atle Lillehaug	Norwegian Veterinary Institute P.O. Box 750 Sentrum N-0106 Oslo Norway	+47 23 21 61 20	atle.lillehaug@vetinst.no
Lone Madsen	Technical University of Denmark National Veterinary Institute Bülowsvej 27 1870 Frederiksberg C Denmark	+45 35 88 68 26	loma@vet.dtu.dk
Werner Wosniok	University of Bremen Institute of Statistics PO Box 330 440 28344 Bremen Germany	+49(421) 218 63784	wwosniok@math.uni-bremen.de

Annex 2: Common and scientific names of host species in the report

abalone, black	*Haliotis cracherodii*
abalone, red	*Haliotis rufescens*
clam, arched razor	*Ensis magnus*
clam, carpet shell	*Ruditapes decussatus*
clam, grooved razor	*Solen marginatus*
clam, Manila	*Ruditapes philippinarum*
clam, pod razor	*Ensis siliqua*
clam, pullet	*Venerupis corrugata*
clam, wedge	*Donax trunculus*
cockle	*Cerastoderma edule*
cod, Atlantic	*Gadus morhua*
cod, Baltic	*Gadus morhua*
cod, Polar	*Boreogadus saida*
crab, blue	*Callinectes sapidus*
dab, common	*Limanda limanda*
dab, long-rough	*Hippoglossoides platessoides*
flounder, European	*Platichthys flesus*
halibut, Atlantic	*Hippoglossus hippoglossus*
herring, Atlantic	*Clupea harengus*
lumpfish	*Cyclopterus lumpus*
minnow	*Phoxinus phoxinus*
mussel, blue	*Mytilus edulis*
mussel, Mediterranean	*Mytilus galloprovincialis*
oyster, Eastern	*Crassostrea virginica*
oyster, European flat	*Ostrea edulis*
oyster, Pacific	*Crassostrea gigas*
salmon, Atlantic	*Salmo salar*
sculpin, shorthorn	*Myoxocephalus scorpius*
trout, brown	*Salmo trutta*
trout, rainbow	*Oncorhynchus mykiss*
whitefish, European	*Coregonus lavaretus*

wrasse, ballan *Labrus bergylta*

RESEARCH ARTICLE

Comparison of infectious agents detected from hatchery and wild juvenile Coho salmon in British Columbia, 2008-2018

Omid Nekouei[1]*, Raphael Vanderstichel[1], Karia H. Kaukinen[2], Krishna Thakur[1], Tobi Ming[2], David A. Patterson[3], Marc Trudel[4], Chrys Neville[2], Kristina M. Miller[2,5]

1 Department of Health Management, University of Prince Edward Island, Charlottetown, PE, Canada, **2** Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, BC, Canada, **3** Fisheries and Oceans Canada, Cooperative Resource Management Institute, School of Resources and Environment Management, Simon Fraser University, Burnaby, BC, Canada, **4** St. Andrews Biological Station, Fisheries and Oceans Canada, St. Andrews, NB, Canada, **5** Forest and Conservation Sciences, University of British Columbia, Vancouver, BC, Canada

* onekouei@upei.ca

Citation: Nekouei O, Vanderstichel R, Kaukinen KH, Thakur K, Ming T, Patterson DA, et al. (2019) Comparison of infectious agents detected from hatchery and wild juvenile Coho salmon in British Columbia, 2008-2018. PLoS ONE 14(9): e0221956. https://doi.org/10.1371/journal.pone.0221956

Editor: Andreas C. Bryhn, Swedish University of Agricultural Science, SWEDEN

Received: February 20, 2019

Accepted: August 19, 2019

Published: September 3, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. The complete raw data are available at http://soggy.zoology.ubc.ca:8080/geonetwork/srv/api/records/97b7f332-51a1-4c78-a695-a57616eb51f3.

Funding: This work was funded by Genome British Columbia, Pacific Salmon Foundation, Pacific Salmon Commission, Bonneville Power Administration, and the Mitacs project (IT06621)

Abstract

Infectious diseases are potential contributors to decline in Coho salmon (*Oncorhynchus kisutch*) populations. Although pathogens are theoretically considered to pose higher risk in high-density rearing environments like hatcheries, there is no direct evidence that hatchery-origin Coho salmon increase the transmission of infectious agents to sympatric wild populations. This study was undertaken to compare prevalence, burden, and diversity of infectious agents between hatchery-reared and wild juvenile Coho salmon in British Columbia (BC), Canada. In total, 2,655 juvenile Coho salmon were collected between 2008 and 2018 from four regions of freshwater and saltwater in BC. High-throughput microfluidics qPCR was employed for simultaneous detection of 36 infectious agents from mixed-tissue samples (gill, brain, heart, liver, and kidney). Thirty-one agents were detected at least once, including ten with prevalence >5%. *Candidatus Brachiomonas cysticola*, *Paraneuclospora theridion*, and *Parvicapsula pseudobranchiocola* were the most prevalent agents. Diversity and burden of infectious agents were substantially higher in marine environment than in freshwater. In Mainland BC, infectious burden and diversity were significantly lower in hatchery smolts than in wild counterparts, whereas in other regions, there were no significant differences. Observed differences in freshwater were predominantly driven by three parasites, *Loma salmonae*, *Myxobolus arcticus*, and *Parvicapsula kabatai*. In saltwater, there were no consistent differences in agent prevalence between hatchery and wild fish shared among the west and east coasts of Vancouver Island. Although some agents showed differential infectious patterns between regions, annual variations likely contributed to this signal. Our findings do not support the hypothesis that hatchery smolts carry higher burdens of infectious agents than conspecific wild fish, reducing the potential risk of transfer to wild smolts at this life stage. Moreover, we provide a baseline of infectious agents in juvenile Coho salmon that will be used in future research and modeling potential correlations between infectious profiles and marine survival.

under the Strategic Salmon Health Initiative and the Salish Sea Marine Survival Program, and partly funded by the Canada Excellence Research Chair in Aquatic Epidemiology. There was no additional external funding received for this study.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Coho salmon (*Oncorhynchus kisutch*) is one of seven Pacific salmon species with cultural, recreational, and economic significance to the residents of the North East Pacific region, especially in Canada [1]. Distinct populations of Coho salmon are found in the coastal streams and rivers of British Columbia (BC) and most of them have a three-year lifecycle [1,2]. Wild Coho salmon populations in the United States and Canada have been in decline for over three decades [3–5]. For instance, in the Interior Fraser River watershed, the abundance of Coho salmon have decreased by more than 60% since 1996, with marine survival reaching an all-time low of 0.1–0.3% in 2014 [2]. Returns for some of Coho salmon populations are so low that they have been listed as endangered or threatened by the Committee on the Status of Endangered Wildlife in Canada or through the Endangered Species Act in the United States [2,6]. Several factors are believed to have contributed to population declines, including climate change, food availability in the marine environment, infectious diseases, predation, fishing, land-use activities, and synergistic effects among these factors [4,5,7].

To increase the abundance of Coho salmon and enhance fishing opportunities along the coast of Canada, the Salmonid Enhancement Program was established in 1970s by Fisheries and Oceans Canada (DFO) and hatchery production of these species was initiated [8]. Hatcheries were considered to be effective because the egg-to-smolt survival in hatcheries was significantly higher than that in wild stocks [9]. However, in the marine environment, lower survival of hatchery-origin fish compared to wild fish has been reported [10]. It has been suggested that due to domestication, hatchery fish generally show reduced swimming ability [11] and lower resistance to stress and diseases than their wild counterparts [12]. There is even evidence that a single generation of hatchery production can reduce the genetic fitness of wild fish [13,14]. Given these findings, the continued use of enhancement hatcheries to produce large numbers of fish for exploitation has been debated. Genetic introgression, overcrowding, competition, predation, predator attraction, and transfer of pathogens and disease are all factors that may carry negative consequences from hatchery to wild fish [15–17]. Although infectious diseases are theoretically considered to pose higher risk in high-density rearing environments like hatcheries, there is still no study showing that hatchery-origin Coho salmon increase the transmission of infectious agents to sympatric wild populations [16,18]. Infectious diseases can disrupt salmon's normal behaviour and physiological performance (e.g. swimming and visual acuity), immunological function, feeding and growth, and can cause mortality in severe cases [7,19]. There is a clear knowledge gap regarding pathogens that can adversely affect the performance and survival of Coho salmon. Out-migrating juveniles are particularly vulnerable to environmental stressors, including infectious agents, during their early marine life, and >90% of them may die in this limited period [4,20,21].

High rearing densities in hatchery environments increase the potential for enhanced transmission of pathogens, but the use of antibiotics and other mitigation measures, such as broodstock selection to minimize vertical transmission of *Renibacterium salmoninarum*, may reduce the incidence and spread of diseases. Alternatively, as many hatcheries use ground rather than river water, hatchery fish may be less exposed to myxozoan parasites that have an alternate invertebrate host in natural freshwater systems. Previous research by our group suggested that naturally occurring myxozoan parasites may be a risk for wild salmon in the ocean [22,23]. Given the observed lower survival of hatchery fish compared to wild fish in the ocean [4], if infection is driving this difference, we would expect that hatchery fish be more vulnerable to infection. As such, we undertook the present cross-sectional study to test the hypothesis that hatchery-reared Coho salmon smolts carry a higher burden of infectious agents at the time they are released from the hatchery compared to their wild counterparts, and that they

continue to carry higher agent burdens in the early marine environment. To test this hypothesis, we applied a high throughput microfluidics system to detect and quantitate 36 infectious agents in juvenile Coho salmon sampled in BC, and compared the prevalence, diversity, and overall infection burden of detected agents between hatchery-origin and wild fish over the last 11 years (2008–2018).

Materials and methods

The animal care and use protocol for this work was approved by the DFO (Fisheries and Oceans Canada) Pacific Region Animal Care Committee (Animal Use Protocol Number: 13–008).

Sample collection

Sampling of juvenile Coho salmon was carried out between 2008 and 2018 (11 year-classes), over the first nine months of early migration. In freshwater, sampling of wild fish was carried out using beach seining within natal lakes or dip netting at smolt fences, generally before hatchery releases. In hatcheries, fish were sampled just prior to release. In the marine environment, fish were collected via mid-water trawl sampling from the Canadian Coast Guard vessel, WE Ricker, or by trawl or purse seine sampling from smaller fishing vessels contracted by DFO. On the trawl vessels, fish were kept on ice and processed within 30 minutes of being brought on-deck. On purse seine vessels, samples were placed in a holding tank on deck until processed. In general, all fish propagated artificially from eggs and milt from the group of spawners and maintained in a controlled hatchery environment until their release are defined as "hatchery fish". Wild fish are defined as fish that developed from eggs produced by parents that spawned naturally in the river bed. In the marine environment, hatchery-origin and wild juvenile Coho salmon were identified as marked and unmarked (with and/or without adipose fin clips) by visual assessment, and/or fish that carried a coded wire tag (CWT). While all clipped and/or CWT fish were known hatchery-origin, there was a slight chance of misclassifying hatchery as wild fish due to different reasons, such as failure in marking, partial clips, or the wire tag was not readily detectable. In addition, in systems whereby hatcheries release fry into lakes (as fry are too small to clip or tag), these would be indistinguishable from wild salmon. However, since these fish rear naturally, we would expect them to closely resemble wild fish with respect to their potential exposure to infectious agents.

Upon sampling, live fish were euthanized in MS-222, assigned a unique identification code, and five tissues (gill, whole brain, heart, liver, and kidney) were sampled aseptically from each fish and preserved in RNAlater (Qiagen, MD, USA). Tissue samples in RNAlater were kept at 4˚C for 24 hours, and transferred to -20˚C for short-term or -80˚C for long-term storage until further processing. Additional fish collected at sea and most freshwater collections were frozen in dry ice and moved to -80˚C until further processing in the laboratory. Overall, 2,655 juvenile Coho were used in our study, including fish sampled in freshwater drainages in the BC Mainland (i.e. Fraser River watershed and central coast) and Vancouver Island, and marine samples within the Salish Sea on the inside waterway between Vancouver Island and the Mainland, and on the west coast of Vancouver Island (Fig 1).

Laboratory analysis

The laboratory methods presented herein have been summarized/adapted from a recent publication by the corresponding author of the present manuscript [24].

Sampled tissues were individually homogenized in TRI-reagent (Ambion Inc., Austin, TX, USA) before extraction. '1-bromo-3-chloropropane' was added to the homogenate, which was then centrifuged to separate aqueous and organic phases. Equal volumes of the aqueous phase

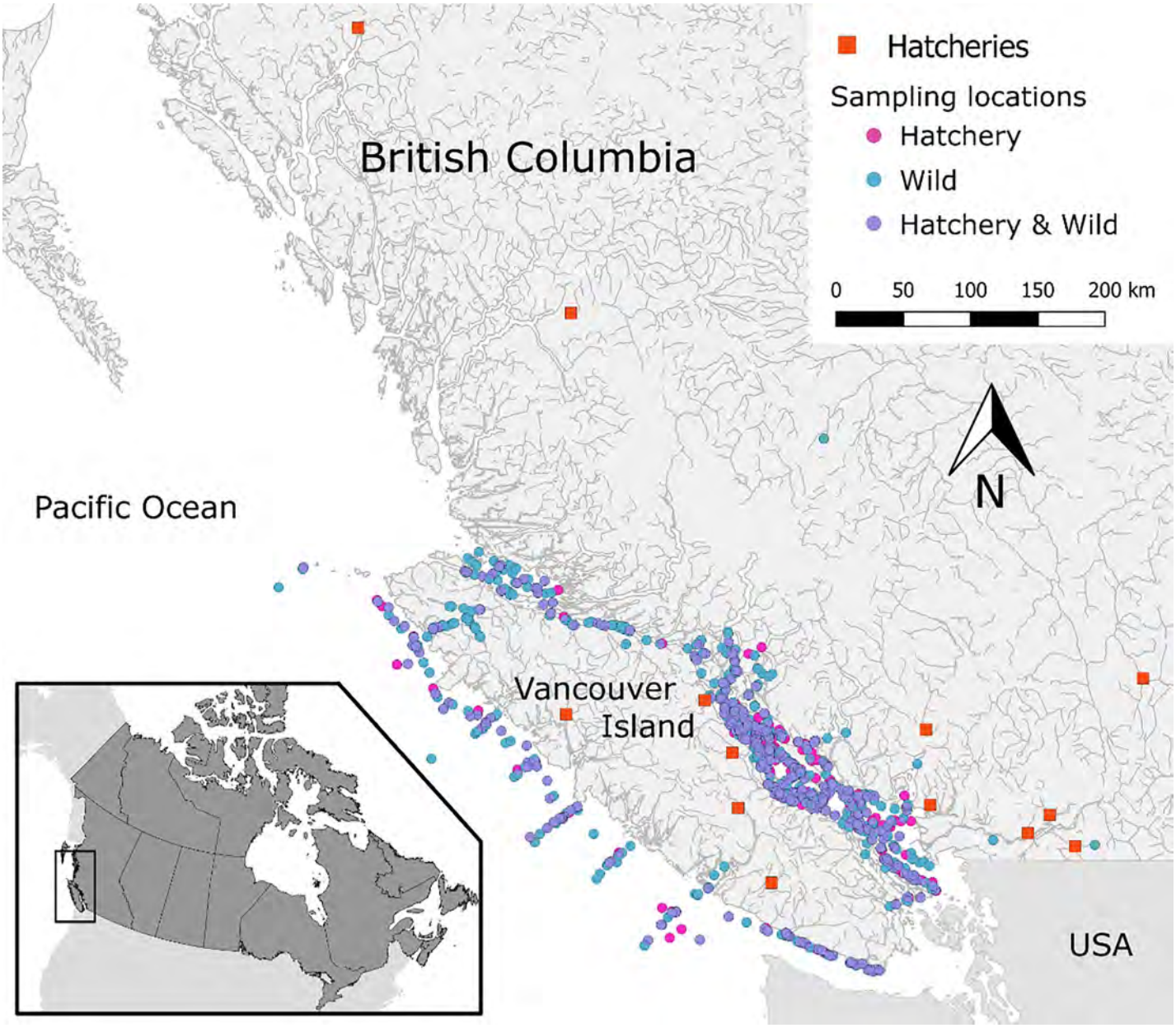


Fig 1. Map of British Columbia, Canada, illustrating sampling locations for 2,655 juvenile Coho salmon by their origin (hatchery based or wild), between 2008 and 2018. This map was created in QGIS v2.18.13 (http://www.qgis.org).

https://doi.org/10.1371/journal.pone.0221956.g001

for each tissue within a fish were combined and placed into a 96-well plate for RNA extraction. Total RNA was extracted from each sample using the MagMax-96 for Microarrays Total RNA Isolation Kit (Ambion Inc.) on a Biomek NXP automated liquid-handling instrument using the 'spin method' protocol. RNA quantity and quality were assessed by measuring the A260/280/230 on a Beckman Coulter DTX 880 Multimode Detector (Brea, CA, USA), and samples diluted to 62.5 ng/μL. RNA (1 μg) was reverse-transcribed to cDNA using SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA) as per the manufacturer's protocol.

DNA was isolated from the organic/interphase portion of TRI-reagent using a high salt TNES-urea buffer [25], followed by the BioSprint 96 DNA extraction kit on a BioSprint 96

workstation (Qiagen, MD). DNA quantity and quality were assessed by measuring the A260/280 on a Beckman Coulter DTX 880 Multimode Detector (Brea, CA, USA), and normalized to 62.5 ng/μL. Equal volumes of DNA and cDNA were combined and used as template for the qPCR assays.

Combined multi-tissue samples of DNA/cDNA for each fish were assessed for the presence and load (abundance) of our infectious agents of interest. Assays were run in duplicate on the Fluidigm BioMark^{TM} HD platform (Fluidigm, South San Francisco). The platform capacity is 96 assays on 96 samples at once (9,216 individual reaction chambers). Assays assessed within the current study included 36 agents in duplicate, and one housekeeping gene (endogenous control to assess RNA quality). The assays included 18 parasites, 12 bacteria, and 6 viruses (Table 1).

On each dynamic array, there were 80 samples and 16 controls run. Negative controls included two negative processing controls for RNA/DNA extraction, two no-template controls for template enrichment (described below), two cDNA (no reverse transcriptase) controls, and two no-template controls for PCR. Positive controls included duplicates of a pooled sample from the cDNA/DNA for all fish used in the study and six serial dilutions of APC clones for all assays to both assess assay integrity and to calculate the copy number of each detected agent; APC clones were loaded last to minimize the potential for contamination. The APC clones were synthesized and cloned sequences of the amplicon for each assay contained an "extra" probe sequence so that potential contamination of high concentration APCs in sample wells could be identified.

BioMark microfluidics qPCR was performed as described in Miller et al. (2016) [26]. Briefly, minute assay volumes (7 nL) are used; therefore, necessitating a pre-amplification step of assays to optimize sensitivity. Dilute primer pairs for each of the 48 assays were combined with TaqMan Preamp MasterMix (Applied Biosystems, Foster City, California) for a final concentration of 50 nM in a 5 μL reaction, and run through 14 cycles of amplification, according to the BioMark protocol. ExoSAP-IT (Affymetrix, Santa Clara, CA) was used to remove unincorporated primers before the samples were diluted 1:5 in DNA Suspension Buffer (Teknova, Hollister, CA).

A 5 μL sample mix was prepared for each pre-amplified sample with TaqMan Universal Master Mix (Life Technologies), GE Sample Loading Reagent (Fluidigm), and a 5 μL aliquot of assay mix was prepared containing 10 μM primers and 3 μM probes for each separate TaqMan assay. An IFC controller HX pressurized and mixed the assays and samples from their individual inlets on the chip. PCR conditions were: 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 s, and 60˚C for 1 min on the BioMark Dynamic Array. Cycle threshold (Ct) was determined using the BioMark Real-Time PCR analysis software. Visual evaluation identified abnormal curve shapes, presence of APC contamination, and correlation of replicates.

For each infectious agent assay in this study, the analytically validated limit of detection (LOD) [26] was applied to the average Ct values (from duplicate qPCR assays) to categorize the test results as either positive or negative. These dichotomized results were further used in the calculation of prevalence and other statistical analyses. The limit of detection is defined as the estimated Ct number under which a given assay is expected to provide true positive results in 95% of the times [26]. If an infectious agent assay detected a Ct signal in only one of the two replicates, the sample was considered 'inconclusive' for that assay and treated as a missing value in our final analyses. While this platform has been proved to provide reliable, rapid, and inexpensive quantitative data on microbe presence and load, its performance for diagnostic purposes in salmon has not been evaluated; however, it has already been applied for human viral and bacterial diagnostics and water quality testing [26]. As such, calculated prevalence for each infectious agent should be interpreted as the test prevalence for the detection of that

Table 1. Thirty-six infectious agents tested on 2,655 juvenile Coho salmon, between 2008 and 2018, along with their overall prevalences (%). The 31 detected agents are presented in the decreasing order of prevalence.

Agent	Abbreviation	Type [a]	N	Positive	Prevalence [b]
1. *Candidatus Brachiomonas cysticola*	c_b_cys	B	2,647	2,363	89.3
2. *Paraneuclospora theridion*	pa_ther	P	2,596	1,023	39.4
3. *Parvicapsula pseudobranchiocola*	pa_pse	P	2,584	965	37.3
4. *Loma salmonae*	lo_sal	P	2,624	756	28.8
5. *Parvicapsula minibicornis*	pa_min	P	2,596	691	26.6
6. *Myxobolus arcticus*	my_arc	P	2,640	289	10.9
7. *Flavobacterium psychrophilum*	fl_psy	B	2,623	217	8.3
8. 1. Gill chlamydia	sch	B	2,638	202	7.7
9. *Parvicapsula kabatai*	pa_kab	P	2,645	164	6.2
10. *Ceratonova shasta*	ce_sha	P	2,646	144	5.4
11. *Tetracapsuloides bryosalmonae*	te_bry	P	2,648	129	4.9
12. *Ichthyophonus hoferi*	ic_hof	P	2,644	97	3.7
13. Erythrocytic necrosis virus	env	V	2,641	91	3.4
14. *Tenacibaculum maritimum*	te_mar	B	2,622	85	3.2
15. Piscine OrthoReovirus	prv	V	2,652	79	3.0
16. *Sphaerothecum destructuens*	sp_des	P	2,653	55	2.1
17. *Myxobolus insidiosus*	my_ins	P	2,651	49	1.8
18. *Nanophyetus salmincola*	na_sal	P	2,654	48	1.8
19. *Crybtobia salmonistica*	cr_sal	P	2,655	43	1.6
20. *Kudoa thyrsites*	ku_thy	P	2,649	45	1.7
21. *Facilispora margolisi*	fa_mar	P	2,626	36	1.4
22. *Ichthyophthirius multifiliis*	ic_mul	P	2,571	21	0.8
23. *Renibacterium salmoninarum*	re_sal	B	2,654	16	0.6
24. Viral hemorrhagic septicemia virus	vhsv	V	2,640	18	0.7
25. *Dermocystidium salmonis*	de_sal	P	2,655	17	0.6
26. *Piscichlamydia salmonis*	pch_sal	B	2,569	16	0.6
27. Rickettsia-like organism	rlo	B	2,651	10	0.4
28. *Vibrio salmonicida*	vi_sal	B	2,650	6	0.2
29. *Neoparamoeba perurans*	ne_per	P	2,654	5	0.2
30. *Piscirickettsia salmonis*	pisck_sal	B	2,650	4	0.2
31. Pacific salmon parvovirus	pspv	V	2,651	4	0.2
32. *Yersinia ruckeri*	ye_ruc_glnA	B	2,654	1	0.0
33. *Aeromonas salmonicida*	ae_sal	B	2,655	0	0.0
34. Infectious hematopoietic necrosis virus	ihnv	V	2,655	0	0.0
35. Viral encephalopathy & retinopathy virus	ver	V	2,655	0	0.0
36. *Vibrio anguillarum*	vi_ang	B	2,655	0	0.0

[a]Type of agent: B = Bacterium, V = Virus, P = Parasite

[b]Overall 'prevalence' was defined as the number of test-positive samples divided by the total number of samples tested for each given infectious agent with conclusive results (i.e. positive / N).

https://doi.org/10.1371/journal.pone.0221956.t001

agent. Due to the use of mixed-tissue samples (including gill), the detection prevalence may not necessarily be equivalent to the prevalence of systemic infection with a given infectious agent, especially in cases with borderline Ct-values. A formal analytical evaluation of our screening platform for application in microbe surveillance research has been implemented and published in 'Miller et al., 2016', including all technical details and analytical validation procedures [26].

Statistical analysis

The final dataset included 2,655 juvenile Coho salmon. All statistical analyses were carried out in Stata v15.1 (StataCorp, College Station, TX, USA).

To effectively compare infectious agents' profiles between hatchery-origin and wild fish in our study, sampling locations were categorized into four main geographical regions, including: (1) freshwater Mainland BC, (2) freshwater Vancouver Island (VI), (3) saltwater/marine on the east coast of VI, also known as the Salish Sea, and (4) saltwater/marine on the west coast of VI. These geographic separations represent the distinct environments encountered along the migration path of the fish. Fig 1 displays the localization of captured fish in BC, colored by the origin of fish (hatchery or wild). This map was created using coordinates of the sampling locations in QGIS v2.18.13 (http://www.qgis.org).

The term 'prevalence' for each infectious agent was defined as the number of fish with the agent detected (positives) divided by the total number of fish tested with conclusive results for that agent. All of the 46 infectious agents and their overall prevalence of detection are reported in Table 1.

The term 'diversity' for each sampled fish was defined as the sum of all infectious agents detected from that sample (i.e. the number of co-detections per fish). A mixed-effects Poisson regression model was built to compare the diversity of infectious agents between hatchery-origin and wild fish within the four sampling regions, including the random effects of year (the outcome of interest was the number of co-detections).

The term 'relative infection burden' (RIB) is a composite metric of multiple infectious agent burden using qPCR data, which was calculated from the following formula:

$$\sum_{i\in m}^{m} \frac{Li}{Lmaxi}$$

Where for a given fish, RNA copy number of the i'th positively-detected infectious agent (*Li*) is divided by the maximum RNA copy number within the population for the i'th infectious agent (*Lmaxi*), and then summed across all detected agents from that fish [27]. A mixed-effects linear regression model was built to compare the RIB of agents between hatchery-origin and wild fish within the four sampling regions, including the random effects of year (the outcome of interest was $\log_{10}$-RIB).

After determining the overall prevalence of all infectious agents, those with a prevalence $> 5\%$ (hereafter, the common agents = the top 10 agents in Table 1) were selected for further statistical analyses. The reason for this selection was to avoid zero counts and/or extremely unbalanced distributions of positive samples (at the eight region×origin combinations).

Mixed-effects logistic regression models were built to compare the prevalence of the common infectious agents between hatchery-origin and wild fish within the four sampling regions. In these analyses, presence or absence of a particular agent within a sample/fish served as the outcome of interest and the random effects of year were included. Bonferroni correction was applied to the significance level (0.05) in multiple comparisons after each model.

Results

Overall, 1,116 and 1,539 juvenile Coho salmon with hatchery and wild origins (respectively) were captured and analyzed in our study. The names and locations of the 13 freshwater hatcheries sampled in this study are indicated in S1 Table. Of 2,655 samples, 23.5% were from freshwater and 76.5% from saltwater. The frequency distribution of the sampled fish by sampling

Table 2. Frequency distribution of 2,655 collected juvenile Coho salmon samples, by sampling region, year-class, and origin (H: hatchery or W: wild). Four sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI.

	FW Main		FW VI		SW East		SW West		
Year	H	W	H	W	H	W	H	W	Total
2008	- [a]	30	8	-	-	4	-	9	51
2009	-	14	-	-	9	6	13	57	99
2010	-	-	-	-	21	78	26	96	221
2011	-	3	-	-	50	78	14	50	195
2012	-	-	-	-	10	54	10	61	135
2013	130	-	156	-	70	128	35	68	587
2014	124	5	-	36	143	203	10	20	541
2015	-	39	18	31	43	125	49	45	350
2016	-	31	-	-	125	118	1	2	277
2017	-	-	-	-	20	62	-	-	82
2018	-	-	-	-	31	86	-	-	117
Total	254	122	182	67	522	942	158	408	2,655

[a] dash indicates no sampling event.

https://doi.org/10.1371/journal.pone.0221956.t002

region, origin, and year-class is presented in Table 2. As shown in this table, freshwater sampling was not carried out from hatchery and wild fish in all years.

Of the 36 infectious agents investigated within each individual fish sample, 31 were detected at least once (Table 1). Ten infectious agents had an overall prevalence > 5% (i.e. the common agents). *Candidatus Brachiomonas cysticola* (c_b_cys) was observed at the highest overall prevalence (89.3%), detected in 2,363 out of 2,647 samples with conclusive results, followed by *Paraneuclospora theridion* (pa_ther, also known as *Desmozoon lepeophtherii*; 39.4%) and *Parvicapsula pseudobranchiocola* (pa_pse; 37.3%). Five infectious agents were not detected at all in our samples. To improve clarity in visualization, only abbreviations for the common infectious agents are used in the Figs (see key in Table 1).

Diversity

The frequency distribution of the diversity of detected agents (co-detections) from each fish by sampling region and origin is presented in Fig 2A. Diversity ranged between 0 and 9, with a median of 3. Approximately, 95% of the samples had a diversity of ≤ 5. The results of the respective Poisson model (indicating changes in the predicted number of detected agents by study region and origin) are illustrated in Fig 2B. The diversity of agents from freshwater to saltwater increased substantially. Hatchery and wild fish did not show any significant difference in diversity, except in freshwater samples from Mainland BC (Fig 2B). In the latter region, wild fish had a significantly greater diversity of agents than hatchery fish ($P < 0.001$). The variability in diversity between study years was highly significant ($P < 0.001$).

Relative infection burden

RIB ranged between 0 and 2.06, with an extremely right-skewed distribution; therefore, a logarithmic transformation was applied to RIB values. The frequency distribution of $\log_{10}$-RIB for detected agents by sampling region and origin is presented in Fig 3A. Consistent with the diversity, a significant increase in RIB was observed from freshwater to saltwater. Moreover, RIB was significantly higher in wild than hatchery fish ($P = 0.004$) only in freshwater samples from Mainland BC (Fig 3B).

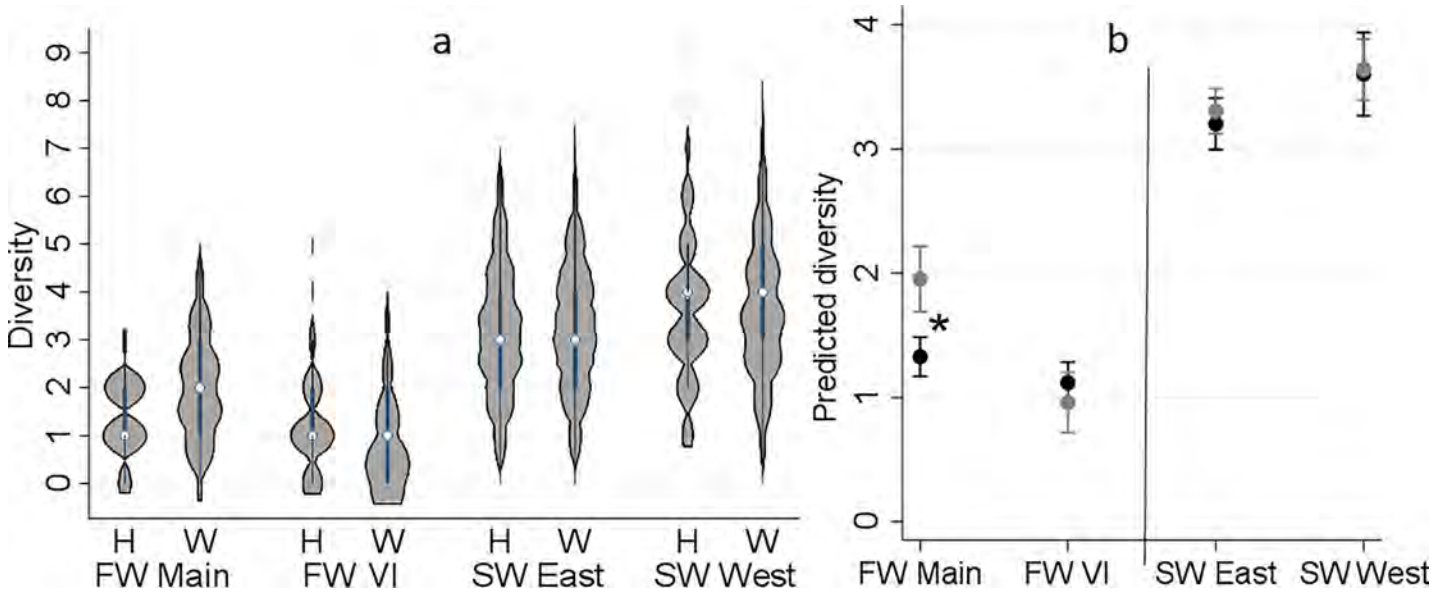


Fig 2. Distribution of the diversity of infectious agents detected in 2,655 juvenile Coho salmon by sampling region and origin (H: hatchery and W: wild) on left side, and the interaction plot for the results of the Poisson regression model, indicating predicted number of detected infectious agents (Y axis) by sampling region and origin (black: hatchery and grey: wild) on right side. Sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. *Statistical comparisons (Bonferroni groups) for predicted diversity between hatchery and wild were only significant in freshwater-Mainland ($P < 0.001$).

https://doi.org/10.1371/journal.pone.0221956.g002

Prevalence of the common agents

Distribution of the prevalences of the common agents by region and origin are presented in Table 3. Among the ten common agents, the following were detected in both freshwater and marine environments: Ca. *B. cysticola*, *Loma salmonae* (lo_sal), *Myxobolus arcticus* (my_arc), and *Parvicapsula kabatai* (pa_kab). *Flavobacterium psychrophilum* (fl_psy) is a known freshwater pathogen and the rest of the prevalent agents were predominantly detected in the marine environment. Due to the scarcity of samples with agent detections for the majority of low-prevalent agents (prevalence <5%; see S2 Table), robust statistical comparison between hatchery and wild fish was not possible; however, the same approach as for the common agents (logistic regression models) was applied for those with a reasonable distribution (1% < prevalence < 5%) in order to provide a general prevalence baseline. Prevalence distributions of the low-prevalent agents are presented in S2 Table. Statistical analyses for the low-prevalent agents did not result in any significant differences in prevalence between hatchery and wild fish ($P > 0.05$ for all; results not shown).

Fig 4 summarizes the results of the logistic regression models for the common agents. In this Fig, relationships of the independent variables of interest (i.e. region and origin, while controlling for the random effects of year) with the predicted probability of infection for any given agent are illustrated. The corresponding table of results is included in the supportive information for reference (S3 Table). A brief description of changes in the prevalence of each common agent with respect to the fish origin is presented below.

The bacterial agent Ca. *B. cysticola* was detected in 89.3% of the samples. As reported in Table 3, its prevalence in marine waters (94.9–98.1%) is substantially higher than in freshwater (20.9–78.7%). The only statistically significant difference between hatchery and wild fish was observed on VI, where prevalence was higher in hatchery fish ($P < 0.001$) (Fig 4).

The microsporidian parasite, *L. salmonae*, was detected in 35% of fish in the marine environment, approximately 3.5× higher than that in freshwater (10%). The only significant

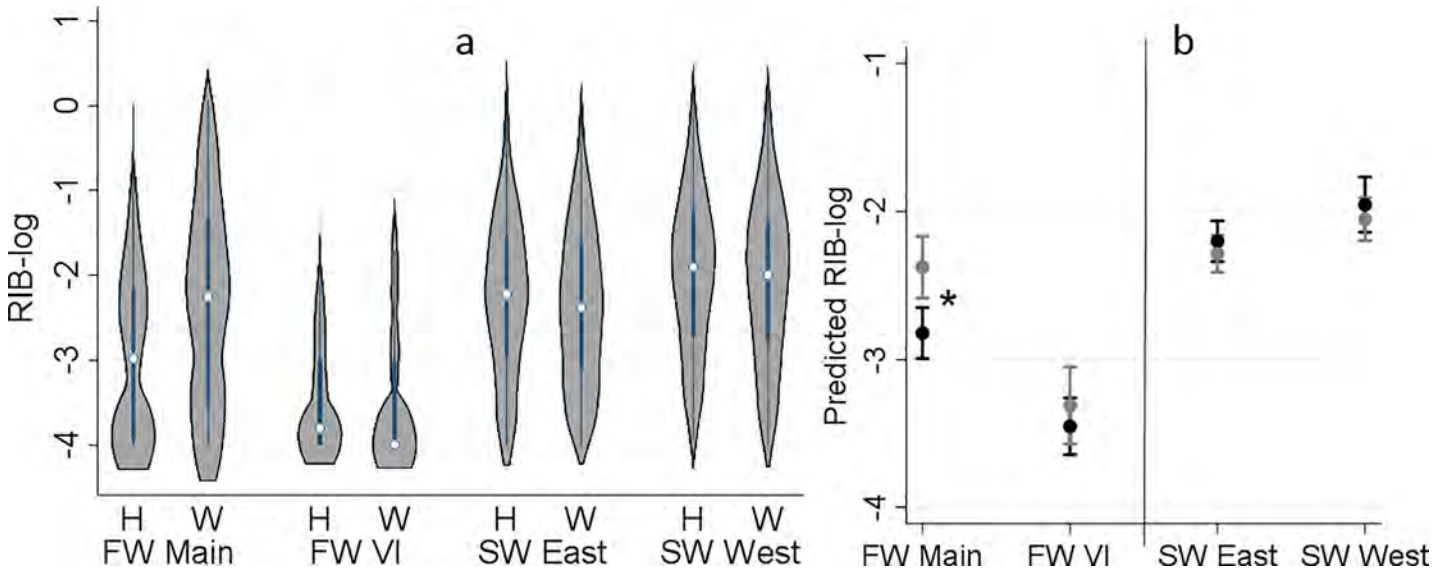


Fig 3. Distribution of log_{10}-relative infection burden (RIB) of all detected infectious agents in 2,655 juvenile Coho salmon by sampling region and origin (H: hatchery and W: wild) on left side, and the interaction plot for the results of the linear regression model, indicating predicted log_{10}-RIB (Y axis) by sampling region and origin (black: hatchery and grey: wild) on right side. Sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. *Statistical comparisons (Bonferroni groups) for predicted RIB-log between hatchery and wild were only significant in freshwater-Mainland ($P < 0.001$).

https://doi.org/10.1371/journal.pone.0221956.g003

difference in the prevalence of this agent between hatchery and wild Coho was observed in freshwater samples from the Mainland BC, where prevalence was higher in wild fish ($P < 0.001$; Fig 4).

The freshwater myxozoan parasite, *M. arcticus*, was consistently more prevalent in wild fish than in hatchery fish in all regions (Fig 4), with statistical significance in all but the freshwater VI samples ($P = 0.712$). Similarly, the overall prevalence of the myxozoan, *P. kabatai*, was higher in wild fish (vs. hatchery fish) in all regions, but the differences were not statistically significant (Fig 4).

The bacterium, *F. psychrophilum*, was only detected in freshwater. Its prevalence in the freshwater Mainland BC samples was significantly higher in hatchery fish than in wild fish ($P = 0.001$) whereas the opposite was true for VI ($P = 0.009$) (Fig 4).

Among the marine agents, microsporidian *P. theridion* and myxozoan *P. pseudobranchiocola* showed similar patterns, with significantly higher prevalence in wild than hatchery fish in

Table 3. Prevalence (%) and the range of samples tested (N) for the prevalent infectious agents from 2,655 juvenile Coho salmon, by sampling region and origin (H: hatchery or W: wild). Four sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. For infectious agents' complete names, refer to key in Table 1.

Region		Origin	N	c_b_cys	pa_ther	pa_pse	lo_sal	pa_min	my_arc	fl_psy	sch	pa_kab	ce_sha
FW	Main	H	249–254	78.7	0.0	0.0	0.4	0.0	4.3	47.4	0.0	0.0	0.0
		W	120–122	77.9	0.8	0.0	27.3	1.6	31.1	30.6	0.0	6.7	0.0
	VI	H	177–182	62.6	0.0	0.0	12.2	0.0	1.7	10.7	0.0	14.8	0.0
		W	62–67	20.9	0.0	0.0	11.9	0.0	7.5	24.2	0.0	18.2	0.0
SW	East	H	499–521	97.1	44.1	40.8	34.0	41.5	6.2	1.4	12.6	2.7	7.5
		W	906–940	94.9	51.5	47.7	35.8	38.5	15.9	1.9	6.7	4.1	4.6
	West	H	153–158	98.1	54.2	57.3	31.4	29.2	1.9	0.6	12.7	4.4	18.4
		W	396–407	95.8	61.3	60.4	33.8	20.6	11.9	0.5	13.5	14.3	8.1

https://doi.org/10.1371/journal.pone.0221956.t003

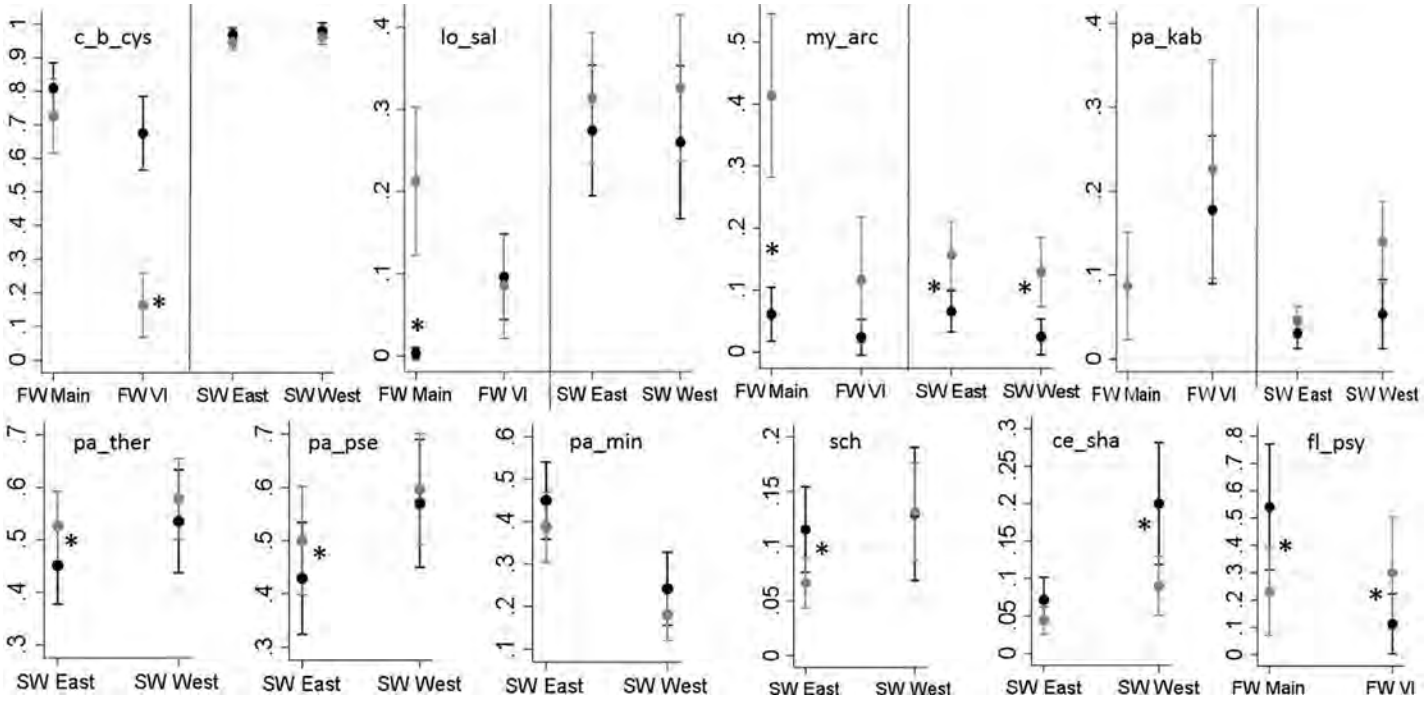


Fig 4. Predicted probabilities of the detection of the prevalent infectious agents (Y axes) in 2,655 juvenile Coho salmon by sampling region (X axes) and origin (black: hatchery and grey: wild), based on logistic regression models. Sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. Note that the axes of these graphs are scaled to maximize resolution between hatchery and wild fish. *Significant statistical comparisons (Bonferroni groups) for predicted probability of detection between hatchery and wild, within each category of region, are indicated with asterisks ($P < 0.05$).

https://doi.org/10.1371/journal.pone.0221956.g004

the marine environment on the east coast of VI (with P = 0.039 and P = 0.047, respectively) (Fig 4). Both agents had approximately 10% higher prevalence on the west coast of VI compared to the east coast of VI (Table 3).

The overall prevalence of myxozoan parasite, *Parvicapsula minibicornis*, was substantially higher (by 16.5%) in fish sampled in marine waters off the east coast of VI than the west coast of VI. There were no significant differences in the prevalence between hatchery and wild fish within each region (Fig 4).

The bacterium, gill chlamydia (sch), was detected at significantly higher prevalence in hatchery-origin fish than in wild fish only in the marine waters off the east coast of VI (P = 0.008; Fig 4). Prevalence off the west coast of VI was 4.5% higher than the east coast of VI (Table 3).

On the west coast of VI, the prevalence of *Ceratonova shasta* (ce_sha) was significantly higher in hatchery-origin fish than in wild fish (P = 0.005; Fig 4).

The results for all pairwise comparisons made between hatchery and wild fish in our study (based on all statistical models) are presented in the supportive information for reference (S4 Table).

Discussion

The three decade-long decline of Coho salmon populations in Canada and the United States [3,4,28] has resulted in the recent listings of multiple populations as endangered or of concern. This issue puts increasing pressure on the scientific community to find the potential causes and identify viable mitigation measures to prevent the extirpation of Coho salmon from the southernmost end of its distribution. Our research is the first comprehensive survey of a broad

range of infectious agents in Coho salmon, and provides a necessary baseline for follow-up studies on infectious agents that may cause diseases and adversely affect the survival and productivity of Coho salmon stocks. Importantly, our data did not support the hypothesis that hatchery fish carry higher diversity and burden of infectious agents than wild fish. While there were some significant differences in the prevalences of specific pathogens between hatchery and wild Coho salmon within different regions, most of these differences were not consistent across all regions. In agreement with our conclusions, Meyers (2005) in a review of hatchery practices in Alaska for controlling two indicator pathogens (infectious hematopoietic necrosis virus, IHNV, and *Renibacterium salmoninarum*) concluded that hatchery practices or hatchery-derived fish did not increase the pathogen levels or prevalences among sympatric wild salmonid stocks [29].

Overall diversity and burden

Diversity and burden of infectious agents in juvenile Coho salmon were substantially higher in the marine environment than in freshwater, consistent with recent studies on juvenile Chinook and Sockeye salmon [22,24,30]. The infectious burden and diversity of detected agents did not show a consistent distinction between hatchery and wild fish in our study. Rather, while divergence was observed in freshwater (with higher levels in wild than hatchery fish), diversity and infectious burden converged in the marine environment. These finding were consistent with a recent study carried out by our team on juvenile Chinook salmon, whereby differences in the infectious profiles between wild and hatchery juvenile Chinook from the Cowichan River system gradually faded as fish converged in the marine environment and migrated along the coast of BC [30]. Herein, difference between hatchery and wild fish in freshwater were predominantly driven by three parasites, *L. salmonae*, *M. arcticus*, and *P. kabatai* (described below). The latter two are myxozoans that require an alternate invertebrate host to complete their life-cycles. Because many hatcheries in the study region utilize ground water rather than river water for rearing, these differences can be explained by exposure to natural sources of infection. The significantly higher prevalences of *L. salmonae* and *M. arcticus* in wild fish (compared to hatchery fish) in Mainland BC were mainly influenced by the spring of 2016 samples, with detections in 97% and 74% of wild fish, respectively, while the prevalences of these two agents in Mainland BC over all other years did not exceed 5% and 33%, respectively. Average spring air temperatures for 2016 were the warmest on record since 1948 in most parts of Mainland BC [31,32], which may have contributed to the high prevalence of these pathogens in wild fish. *L. salmonae* xenoma development is known to be temperature dependent, with permissive temperatures between 9 and 20 ^{o}C [33,34]. Our analyses also showed that the variability between study years was highly significant. However, we were not able to evaluate the prevalence of infectious agents on a yearly basis due to the lack of freshwater samples from several years.

Prevalent infectious agents

Distributions of the common agents in our study locations were quite variable on an agent-by-agent basis; hence, we briefly discuss each agent, individually.

Loma salmonae is a microsporidian pathogen of salmonid fish which can cause salmonid microsporidial gill disease, SMGD, in BC [35]. While *L. salmonae* has been detected from wild and farmed Chinook salmon at high levels of prevalence [22,36], it was rarely detected in juvenile migratory Sockeye and farmed Atlantic salmon in BC [24,36]. Coho salmon appears to be sensitive to the infection but less sensitive than Chinook [34]. As explained above, 2016 samples drove the high prevalence of this agent in wild fish from Mainland BC.

M. arcticus is a myxozoan parasite, endemic to BC, which we have detected across a range of Pacific salmon species [22,24,30]. *M. arcticus* is known to infect juvenile salmon in freshwater [37]. The prevalence of *M. arcticus* in wild fish was consistently higher than in hatchery fish in all study locations. This finding may be due to the primary use of ground water in hatcheries, which presumably would not carry its annelid intermediate host (*Stylodrilus heringianus*). However, some exposure could occur when hatcheries sporadically switch to natural river water for rearing. The substantially higher prevalence of this agent in Mainland BC is likely associated with the abundance of its intermediate host, especially in the Fraser River system [38]. While *M. arcticus* has been associated with abnormal swimming behavior in naturally infected smolts [39], it appears to cause negligible direct pathological effects [40].

P. kabatai was first detected from adult pink salmon in Quinsam River [41]. In this study, we detected this parasite at low prevalence (overall, 6.2%) from both freshwater and saltwater samples. The intermediate host for *P. kabatai* is unknown, but it must reside in freshwater given the detection of this parasite in freshwater. This agent has recently been detected across other salmon species [22,24,30,36]. *P. kabatai* has been associated with increased likelihood of predation of juvenile Sockeye salmon by *Rhinoceros Auklets* [7]. There is still no information on the potential pathogenic effects of this agent in Pacific salmon species, which demands further research.

Since its first report in BC salmon in 2014, Ca. *B. cysticola* has consistently been the most frequent infectious agent detected in Pacific salmon species in both fresh and saltwater habitats in BC, typically with a prevalence $>$ 80% [7,22,24,30]. Our findings in juvenile Coho salmon are consistent with other Pacific salmon species; however, wild juveniles sampled in freshwater systems on VI showed significantly lower prevalence (21%) than in other locations. A possible explanation for this finding may be the reduced abundance of Ca. *B. cysticola* in VI natural habitats, or it could be due to the relatively low number of wild juveniles captured/tested from this location in our study (n = 67). This bacterium is also prevalent in Europe and is associated with proliferative gill inflammation in net pens, but its role in the development of the disease has not yet been established [42,43]. However, Wang (2018) recently showed that in juvenile Chinook salmon, Ca. *B. cysticola* was significantly associated with the elevation of inflammatory response in the gill transcriptome [23].

P. theridion is a microsporidian parasite carried and possibly transmitted by salmon lice in saltwater. This parasite was discovered in farmed Atlantic salmon in western Norway in 2008, and is considered a primary agent in cases with high mortality linked to proliferative gill disease in western Norway [44,45]. This parasite has consistently been detected with high prevalences in seawater from farmed and wild salmon species in BC [22,24,30,36]. It has been shown that hatchery juvenile Coho salmon are fairly resistant to salmon lice [46]. On average, hatchery-origin fish have larger size as compared to their wild counterparts at the time of release, which may explain why we observed lower prevalence of *P. theridion* in our hatchery Coho than in wild fish.

P. pseudobranchiocola is a marine myxozoan parasite which has been detected from other Pacific salmon species in BC, especially Chinook [22,24,30]. The parasite can infect the pseudobranchs of gill tissue and has been associated with gill disease and mortality in farmed salmon [47], as well as impacts on swim performance and visual acuity [48]. *P. pseudobranchicola* was shown to be associated with increased risk of predation of juvenile Sockeye salmon [7]. Although the known life cycles of parvicapsulids involve a polychaete alternate host, the life cycle of *P. pseudobranchicola* is still unknown [49,50]. This parasite was more prevalent in wild Coho samples than in hatchery-origin fish in Salish Sea. One possible explanation for this finding could be longer exposure of wild fish to Seatrout (*Salmo trutta*), which has been identified as a natural host for *P. pseudobranchicola* [50].

P. minibicornis is an endemic freshwater-transmitted myxozoan which has been observed in other Pacific salmon species [22,24,51]. This parasite was the only agent, among the common agents, that had a higher prevalence on the east coast of VI (Salish Sea) than the west coast. This was likely due to the high estuarine abundance of its alternate host, *Manayunkia speciosa*, around Salish Sea. *P. minibicornis* typically causes lesions in kidney tissue, but is also associated with branchitis, osmoregulatory dysfunction, and pre-mature mortality in returning Sockeye in the Fraser River system [52]. *P. minibicornis* has been linked with parasite-associated mortality in freshwater in returning Sockeye salmon [18,22]. Juvenile Sockeye salmon infection with *P. minibicornis* was linked with increased risk of predation by *Rhinoceros Auklets* in the marine environment [7], and associated pathological changes and differential expression of immune genes have been demonstrated in juvenile Chinook salmon sampled in the marine environment [23].

C. shasta is a highly virulent myxozoan parasite that is endemic in salmon-bearing rivers throughout the Pacific Northwest [53]. It causes ceratomyxosis, which is reportable to the Canadian Food Inspection Agency and the World Organisation for Animal Health. The alternate oligochaete host (*Manayunkia speciosa*) resides in freshwater and estuarine environments. *C. shasta* can cause disease in all salmonid fish [54]. Interestingly, while this parasite is only known to be transmitted in freshwater (which is also the only environment in which pathogenicity has been established), our studies have repeatedly observed increasing abundances of this agent in the marine environment [22,24,30]. Higher prevalence of this agent in hatchery fish (compared to sympatric wild fish) in our study was in agreement with findings in Cowichan juvenile Chinook [30]. Given the higher abundance and larger difference between hatchery and wild fish on the west coast, we suspect that an abundance of hatchery fish from the Columbia River could explain this difference, as we know that *C. shasta* is highly prevalent within the Columbia River watershed [55,56]. With respect to the importance of this agent in saltwater, follow-up studies are necessary to elaborate on the pathogenicity and epidemiology of *C. shasta* in Pacific salmon species although there is some evidence of associated pathogenicity in juvenile Chinook salmon sampled in the ocean [23].

Salmon gill chlamydia (sch) was originally detected in farmed Atlantic salmon suffering from proliferative gill disease in Europe [57]. This agent has also been detected from other Pacific salmon species at the similar low to moderate levels of prevalence as in our study [22,24,30,36]. This bacterial agent was more prevalent in hatchery fish than wild fish, but only on the east coast of Vancouver Island. However, this trend was not consistent over the study years. We could not find any reasonable explanation for this finding. There is a clear knowledge gap around the pathogenesis and exact role of gill chlamydia in proliferative gill disease, which demands additional research.

F. psychrophilum is the causative agent of bacterial cold water disease in salmonid fish in freshwater [58]. The prevalence of *F. psychrophilum* in juvenile Chinook and Sockeye salmon in BC has been low (<5%) [22,24,30]. In agreement with the higher prevalence of this agent in our study (8.3%), it has been suggested that Coho salmon are particularly susceptible to the infection, which can lead to high levels of mortality, especially in juveniles [58]. Hatchery Coho from the Mainland BC showed substantially higher prevalence of *F. psychrophilum* compared to wild and VI fish in this study. It appeared that this difference was mostly driven by one Mainland hatchery, Chilliwack River Hatchery, experiencing very high prevalence of this bacterium in the two years of hatchery sampling (2013 and 2014), with detection in 50 out of the 59 samples tested.

Low-prevalent agents

Low-prevalent infectious agents were included in the calculations and analyses of the overall diversity and infection burden. We did not find any prominent differences in the prevalence

of these agents between hatchery and wild fish; nonetheless, we recognize that our power to detect differences for these agents was low. *Tetracapsuloides bryosalmonae*, *Ichthyophonus hoferi*, *Tenacibaculum maritimum*, and *Sphaerothecum destructuens* are all known pathogens in salmonid fish which can cause acute diseases with high levels of morbidity and mortality [7,59]. As a result, these agents may not be tolerated in high abundance, consistent with their observed low prevalences.

Erythrocytic necrosis virus (ENV) is an iridovirus that causes viral erythrocytic necrosis [60] in multiple species [61–63]. ENV is more prevalent in juvenile Sockeye and Chinook salmon [22,24]. It can cause anemia, reduction in stamina, and predispose fish to other infections, and/or increase the impact of other stressors (e.g. low oxygen) and predation, at times leading to population-level impacts in susceptible species [64]. Piscine orthoreovirus subtype-1 (PRV-1) is the causative agent of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon in Norway [65]. PRV-2 is the probable cause of erythrocytic inclusion body syndrome (EIBS) in Coho salmon in Japan [66]. Thus far, only PRV-1 has been detected in BC. Although PRV-1 has been associated with jaundice syndrome in farmed Chinook salmon [67], it has not yet been established as the causative agent of a disease in BC Pacific salmon [68,69].

All of the low prevalent infectious agents seem to be naturally occurring components of freshwater and marine ecosystems. Most of these agents have not been studied in Coho salmon at all and future studies are required to elucidate their potential pathogenic effects and association with survival and productivity of Coho salmon at the population level. In addition, we detected some other important pathogens of salmonids from our samples at prevalences <1%, such as viral hemorrhagic septicemia virus (VHSV) and *Renibacterium salmoninarum*. However, we were not able to assess the distribution of these agents due to very small number of detections; therefore, they stayed beyond the scope of this document. Our future studies will take aim at the most significant pathogens in Coho salmon.

In our recent studies on various Pacific salmon species in BC [7,22,24,30,36], some of the most virulent viral agents that have greatly affected farmed salmonids across the world; e.g., infectious salmon anemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), salmonid alphavirus (SAV), and *Oncorynchus masou* herpesvirus (OMV) were not detected in any samples; therefore, we did not include those in our test panel. Infectious hematopoietic necrosis virus (IHNV) is a very important pathogen in salmonid fish, reportable to the Canadian Food Inspection Agency and the World Organisation for Animal Health. We did not detect this virus from our samples at all. Although IHNV has been detected in adult Coho salmon, it is believed that Coho are not susceptible to the disease [70,71].

Limitations

There were a number of limitations in our study, including: (1) the uncertainty around the origin of a number of captured fish. As explained in the materials and methods section, there was a chance that the hatchery marks (fin clips and/or coded wire tags) were not detectable in hatchery-origin fish and they were misclassified as wild fish. In other words, all fish classified as 'hatchery' are definitely hatchery fish, but not all 'wild fish' were born wild. Therefore, we are more confident in the hatchery results than in wild. However, we believe this issue would have been limited to a small proportion of fish and would not likely bias our general conclusions; (2) some fish captured off the west coast may have originated from the Columbia River system because individual stock identification in Coho salmon was not possible in our study; (3) sampling from freshwater was not conducted in all study years. For instance, all 254 samples from hatcheries located in Mainland BC were collected in 2013 and 2014, while there were

only 5 comparable wild samples available (from this region in 2014). This issue might have decreased the comparability of our freshwater samples (i.e. lack/shortage of appropriate matches); (4) our screening tool, Fluidigm BioMark™ microfluidics qPCR, was used with some limitations (outlined under the laboratory analysis section), which demand caution in the interpretation of the results. For instance, there was a chance to misclassify a positive sample as 'negative' and therefore slightly underestimated the prevalence of an infectious agent when samples with very low RNA copy numbers for that agent were present. The loads of detected infectious agents for hatchery and wild fish, by study region, have been presented in the supplementary materials (S1 and S2 Figs). As shown, general patterns of the loads are consistent with the observed prevalences of common infectious agents.

Although we included random effects of years in our models, future comparisons between hatchery and wild fish in freshwater within each specific year may explain some of the infectious patterns observed in our study.

Although fish size has been shown to be variable among juvenile Coho salmon in freshwater, based on their origin [72], we did not include 'size' (with 160 missing values) in our models because its potential effect was partially captured by the origin of fish (hatchery versus wild). Perhaps, one of the reasons for higher burden and diversity of infectious agents observed in Mainland BC wild fish (compared to their hatchery counterparts) may be their greater susceptibility to some infectious agents due to their overall smaller size [72,73], but the absence of difference between hatchery and wild fish on Vancouver Island contradicts this possibility.

Despite the outlined limitations, we believe our large sample size, collected over an expansive number of years (i.e. 11 years) and over a wide geographical region in BC provides valuable baseline information on the presence and overall distribution of important infectious agents in juvenile Coho salmon, as well as reasonably robust comparisons between hatchery-origin and wild fish.

Conclusions

This study provides a baseline dataset on the detection of infectious agents in wild and hatchery-reared Coho salmon, spanning a decade of sampling efforts. Our study does not support the hypothesis that hatchery fish carry higher diversity and burden of infectious agents compared to sympatric wild salmon species [15–17]. Overall, it appears that wild juvenile Coho from Mainland BC (mostly, originated from Fraser River system) carry higher levels of infections than their hatchery-origin counterparts, largely owing to parasites with alternate invertebrate hosts. While there were some differences between hatchery-origin and wild fish in the marine environment, they were rarely consistent between the east and west coast, suggesting that divergent distributions were not due to differences in susceptibility, but rather potentially explained by differences in habitat use along the coast, stock representation (e.g. Columbia-origin hatchery fish), and representation of hatchery and wild fish in different years. While our study sheds new light on the range of agents that Coho salmon carry, there remains a lot of unknowns regarding which agents have the highest pathogenic potential. Our future research will focus on histopathological evidence of diseases associated with these agents, shifts in agent distributions between seasons, and evaluation of potential associations between infections and marine survival and productivity of major Coho salmon populations.

Supporting information

S1 Fig. Distribution of the loads (log10 (copy number + 1)) of the prevalent infectious agents detected in 2,655 juvenile Coho salmon by sampling region and origin (hatchery: black & wild: white). Sampling regions: 1) freshwater-mainland; 2) freshwater-Vancouver

Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. For infectious agent's complete name, refer to Table 1.
(TIF)

S2 Fig. Distribution of the loads (log10 (copy number + 1)) of the low-prevalent infectious agents (1% < prevalence < 5%) detected in 2,655 juvenile Coho salmon by sampling region and origin (hatchery: black & wild: white). Sampling regions: 1) freshwater-mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. For infectious agent's complete name, refer to Table 1.
(TIF)

S1 Table. Location (M: Mainland and VI: Vancouver Island) and the number of fish (N) from the 13 freshwater hatcheries in the study.
(PDF)

S2 Table. Prevalence (%) and the range of samples tested (N) for the low-prevalent infectious agents (1% < prevalence < 5%) from 2,655 juvenile Coho salmon, by sampling region and origin (hatchery or wild). Four sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI. For infectious agents' complete names, refer to key in Table 1.
(PDF)

S3 Table. Results of the logistic regression models, evaluating the associations between sampling region and origin, and the prevalence of each common infectious agent (dichotomous outcome). Sampling regions: 1) freshwater-Mainland; 2) freshwater-Vancouver Island (VI); 3) saltwater-east coast of VI; and 4) saltwater-west coast of VI.
(PDF)

S4 Table. Pairwise comparisons for all of the models in the study: Diversity, Relative Infection Burdon (RIB-log), and the prevalence of ten common agents. Bonferroni adjustment has been applied to all analyses, including combinations of fish origin (Hatchery or Wild) and sampling regions: 1) freshwater-Mainland (FW Main); 2) freshwater-Vancouver Island (FW VI); 3) saltwater-east coast of VI (SW East); and 4) saltwater-west coast of VI (SW West).
(PDF)

Acknowledgments

We thank Fisheries and Oceans Canada, Genome British Columbia, Pacific Salmon Foundation, Bonneville Power Administration, Pacific Salmon Commission, Mitacs, and Canada Excellence Research Chair in Aquatic Epidemiology for their valuable contributions to this research. We also thank all of the vessel crew, biologists, and technicians who collected the samples on the research and commercial vessels.

Author Contributions

Conceptualization: Omid Nekouei, Raphael Vanderstichel, Kristina M. Miller.

Data curation: Karia H. Kaukinen, Tobi Ming.

Formal analysis: Omid Nekouei.

Funding acquisition: Raphael Vanderstichel.

Investigation: David A. Patterson, Marc Trudel, Chrys Neville, Kristina M. Miller.

Methodology: Omid Nekouei, Raphael Vanderstichel, Karia H. Kaukinen, Krishna Thakur, Tobi Ming, David A. Patterson.

Project administration: Kristina M. Miller.

Resources: David A. Patterson, Marc Trudel, Chrys Neville, Kristina M. Miller.

Software: Omid Nekouei.

Supervision: Raphael Vanderstichel, Kristina M. Miller.

Visualization: Omid Nekouei.

Writing – original draft: Omid Nekouei.

Writing – review & editing: Krishna Thakur, Kristina M. Miller.

References

1. Groot C ML. Pacific Salmon Life Histories. Vancouver: UBC Press; 1991.
2. DFO. In: 2019 [Internet]. Available: http://www.dfo-mpo.gc.ca/species-especes/profiles-profils/salmon-coho-saumon-eng.html
3. Bradford MJ, Irvine JR. Land use, fishing, climate change, and the decline of Thompson River, British Columbia, coho salmon. Can J Fish Aquat Sci. 2000; 57: 13–16. https://doi.org/10.1139/f99-283
4. Zimmerman MS, Irvine JR, O'Neill M, Anderson JH, Greene CM, Weinheimer J, et al. Spatial and Temporal Patterns in Smolt Survival of Wild and Hatchery Coho Salmon in the Salish Sea. Mar Coast Fish. 2015; 7: 116–134. https://doi.org/10.1080/19425120.2015.1012246
5. Brown LR, Moyle PB, Yoshiyama RM. Historical Decline and Current Status of Coho Salmon in California. North Am J Fish Manag. 1994; https://doi.org/10.1577/1548-8675(1994)014<0237:HDACSO>2.3.CO;2
6. NOAA. In: 2019 [Internet]. Available: https://www.fisheries.noaa.gov/species/coho-salmon-protected
7. Miller KM, Teffer A, Tucker S, Li S, Schulze AD, Trudel M, et al. Infectious disease, shifting climates, and opportunistic predators: Cumulative factors potentially impacting wild salmon declines. Evol Appl. 2014; 7: 812–855. https://doi.org/10.1111/eva.12164 PMID: 25469162
8. Hilborn R, Winton J. Learning to enhance salmon production: lessons from the salmonid enhancement program. Can J Fish Aquat Sci. 1993; https://doi.org/10.1139/f93-228
9. Sweeting RM, Beamish RJ, Noakes DJ, Neville C. Replacement of wild Coho Salmon by hatchery-reared Coho Salmon in the Strait of Georgia over the past three decades. North Am J Fish Manag. 2003; https://doi.org/10.1577/1548-8675(2003)023<0492:ROWCSB>2.0.CO;2
10. Beamish RJ, Sweeting RM, Neville CM, Lange KL, Beacham TD, Preikshot D. Wild chinook salmon survive better than hatchery salmon in a period of poor production. Environ Biol Fishes. 2012; https://doi.org/10.1007/s10641-011-9853-8
11. Bams RA. Differences in performance of naturally and artificially propagated sockeye salmon migrant fry, as measured with swimming and predation tests. J Fish Res Board Canada. 1967; https://doi.org/10.1139/f67-094
12. Salonius K, Iwama GK. Effects of early rearing environment on stress response, immune function, and disease resistance in juvenile coho (Oncorhynchus kisutch) and chinook salmon (O. tshawytscha). Can J Fish Aquat Sci. 1993; 50: 759–766. https://doi.org/10.1139/f93-087
13. Araki H, Berejikian BA, Ford MJ, Blouin MS. SYNTHESIS: Fitness of hatchery-reared salmonids in the wild. Evol Appl. 2008; https://doi.org/10.1111/j.1752-4571.2008.00026.x14
14. Araki H, Cooper B, Blouin MS. Genetic effects of captive breeding cause a rapid, cumulative fitness decline in the wild. Science. 2007; https://doi.org/10.1126/science.1145621 PMID: 17916734
15. Weber ED, Fausch KD. Interactions between hatchery and wild salmonids in streams: differences in biology and evidence for competition. Can J Fish Aquat Sci. 2003; https://doi.org/10.1139/f03-087
16. Naish KA, Taylor JE, Levin PS, Quinn TP, Winton JR, Huppert D, et al. An evaluation of the effects of conservation and fishery enhancement hatcheries on wild populations of salmon. Advances in Marine Biology. 2007. https://doi.org/10.1016/S0065-2881(07)53002-6
17. Christie MR, Marine ML, French RA, Waples RS, Blouin MS. Effective size of a wild salmonid population is greatly reduced by hatchery supplementation. Heredity (Edinb). 2012; https://doi.org/10.1038/hdy.2012.39 PMID: 22805657

18. Kent M. Infectious diseases and potential impacts on survival of fraser river Sockeye Salmon. Cohen Comm Tech Rept. 2011; 1: 1–58.
19. Costello MJ. Ecology of sea lice parasitic on farmed and wild fish. Trends Parasitol. 2006; 22: 475–483. https://doi.org/10.1016/j.pt.2006.08.006 PMID: 16920027
20. Beamish RJ, Mahnken C. A critical size and period hypothesis to explain natural regulation of salmon abundance and the linkage to climate and climate change. Prog Oceanogr. 2001; 49: 423–437. https://doi.org/10.1016/S0079-6611(01)00034-9
21. Mckinnell S, Curchitser E, Groot K, Kaeriyama M, Trudel M. Oceanic and atmospheric extremes motivate a new hypothesis for variable marine survival of Fraser River sockeye salmon. Fish Oceanogr. 2014; 23: 322–341. https://doi.org/10.1111/fog.12063
22. Tucker S, Li S, Kaukinen KH, Patterson DA, Miller KM. Distinct seasonal infectious agent profiles in life-history variants of juvenile Fraser River Chinook salmon: An application of high-throughput genomic screening. PLoS One. 2018; https://doi.org/10.1371/journal.pone.0195472 PMID: 29672620
23. Wang Y. The physiological associations between infectious agents and migrating juvenile Chinook salmon (*Oncorhynchus Tshawytscha*). M.Sc. Thesis, The University of British Columbia. 2018. Available: https://open.library.ubc.ca/cIRcle/collections/ubctheses/24/items/1.0375894
24. Nekouei O, Vanderstichel R, Ming T, Kaukinen KH, Thakur K, Tabata A, et al. Detection and assessment of the distribution of infectious agents in juvenile Fraser River Sockeye Salmon, Canada, in 2012 and 2013. Front Microbiol. 2018; 9: 1–16. https://doi.org/10.3389/fmicb.2018.00001
25. Asahida T, Kobayashi T, Saitoh K, Nakayama I. Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentration of urea. Fish Sci. 1996; 62: 727–730. https://doi.org/10.2331/fishsci.62.727
26. Miller KM, Gardner IA, Vanderstichel R, Burnley T, Angela D, Li S, et al. Report on the performance evaluation of the Fluidigm BioMark platform for high-throughput microbe monitoring in salmon. DFO CSAS Rep. 2016; Research D: 1–282. https://doi.org/10.13140/RG.2.2.15360.84487 Available: http://publications.gc.ca/collections/collection_2016/mpo-dfo/Fs70-5-2016-038-eng.pdf
27. Bass AL, Hinch SG, Teffer AK, Patterson DA, Miller KM. Fisheries capture and infectious agents are associated with travel rate and survival of Chinook salmon during spawning migration. Fish Res. 2019; https://doi.org/10.1016/j.fishres.2018.09.009
28. DFO. Marine survival forecast of Southern British Columbia Coho. DFO Can Sci Advis Sec Sci Advis Rep. 2010;2009/073. Available: http://waves-vagues.dfo-mpo.gc.ca/Library/340356.pdf
29. Meyers T. Disease transmission from cultured salmonids to wild fish stocks: perspectives on the Alaskan hatchery program. 2005. pp. 228–237.
30. Thakur KK, Vanderstichel R, Li S, Laurin E, Tucker S, Neville C, et al. A comparison of infectious agents between hatchery-enhanced and wild out-migrating juvenile chinook salmon (*Oncorhynchus tshawytscha*) from Cowichan River, British Columbia. Facets. 2018; 3: 695–721. https://doi.org/10.1139/facets-2017-0113
31. Environment and Climate Change Canada. Climate Trends and Variations Bulletin–Annual 2015 [Internet]. 2016. Available: http://ec.gc.ca/sc-cs/default.asp?lang=En&n=7150CD6C-1
32. Canadian Meteorological and Oceanographic Society—Publications: Top_Ten [Internet]. 2019 [cited 6 Feb 2019]. Available: https://www.cmos.ca/site/top_ten?a=2016&language=en_CA#twospring
33. Beaman HJ, Speare DJ, Brimacombe M. Regulatory effects of water temperature on *Loma salmonae* (Microspora) development in rainbow trout. J Aquat Anim Health. 1999; https://doi.org/10.1577/1548-8667(1999)011<0237:REOWTO>2.0.CO;2
34. Becker JA, Speare DJ. Transmission of the microsporidian gill parasite, *Loma salmonae*. Anim Health Res Rev. 2007; 8: 59–68. https://doi.org/10.1017/S1466252307001223 PMID: 17692143
35. Kent ML, Speare DJ. Review of the sequential development of *Loma salmonae* (Microsporidia) based on experimental infections of rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*O. tshawytscha*). Folia Parasitol (Praha). 2005; 52: 63–68. https://doi.org/10.14411/fp.2005.009
36. Laurin E, Jaramillo D, Vanderstichel R, Ferguson H, Kaukinen KH, Schulze AD, et al. Histopathological and novel high-throughput molecular monitoring data from farmed salmon (*Salmo salar* and *Oncorhynchus spp.*) in British Columbia, Canada, from 2011–2013. Aquaculture. Elsevier; 2019; 499: 220–234. https://doi.org/10.1016/j.aquaculture.2018.08.072
37. Mahony AM, Johnson SC, Neville CM, Thiess ME, Jones SRM. *Myxobolus arcticus* and *Parvicapsula minibicornis* infections in sockeye salmon *Oncorhynchus nerka* following downstream migration in British Columbia. Dis Aquat Organ. 2017; 126: 89–98. https://doi.org/10.3354/dao03158 PMID: 29044039
38. Kent ML, Whitaker DJ, Margolis L. Transmission of *Myxobolus arcticus* Pugachev and Khokhlov, 1979, a myxosporean parasite of Pacific salmon, via a triactinomyxon from the aquatic oligochaete *Stylodrilus heringianus* (Lumbriculidae). Can J Zool. 1993; 71: 1207–1211. https://doi.org/10.1139/z93-165

39. Moles a., Heifetz J. Effects of the brain parasite *Myxobolus arcticus* on sockeye salmon. J Fish Biol. 1998; 52: 146–151.

40. Urawa S, Freeman MA, Johnson SC, Jones SRM, Yokoyama H. Geographical variation in spore morphology, gene sequences, and host specificity of *Myxobolus arcticus* (Myxozoa) infecting salmonid nerve tissues. Dis Aquat Organ. 2011; 96: 229–237. https://doi.org/10.3354/dao02398 PMID: 22132501

41. Jones S, Prosperi-Porta G, Dawe S. a New Parvicapsulid (Myxosporea) Species in Adult Pink Salmon, *Oncorhynchus Gorbuscha*, From the Quinsam River, British Columbia, Canada. J Parasitol. 2006; 92: 1313–1318. https://doi.org/10.1645/GE-940R.1 PMID: 17304813

42. Toenshoff ER, Kvellestad A, Mitchell SO, Steinum T, Falk K, Colquhoun DJ, et al. A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (*Salmo salar*). PLoS One. 2012; 7: 1–7. https://doi.org/10.1371/journal.pone.0032696 PMID: 22427865

43. Mitchell SO, Steinum TM, Toenshoff ER, Kvellestad A, Falk K, Horn M, et al. *Candidatus branchiomonas cysticola* is a common agent of epitheliocysts in seawater-farmed atlantic salmon salmo salar in norway and ireland. Dis Aquat Organ. 2013; 103: 35–43. https://doi.org/10.3354/dao02563 PMID: 23482383

44. Nylund S, Nylund A, Watanabe K, Arnesen CE, Karlsbakk E. Paranucleospora theridion n. gen., n. sp. (Microsporidia, Enterocytozoonidae) with a Life Cycle in the Salmon Louse (*Lepeophtheirus salmonis, Copepoda*) and Atlantic Salmon (*Salmo salar*). J Eukaryot Microbiol. 2010; 57: 95–114. https://doi.org/10.1111/j.1550-7408.2009.00451.x PMID: 20070452

45. Nylund S, Andersen L, Sævareid I, Plarre H, Watanabe K, Arnesen CE, et al. Diseases of farmed Atlantic salmon Salmo salar associated with infections by the microsporidian *Paranucleospora theridion*. Dis Aquat Organ. 2011; 94: 41–57. https://doi.org/10.3354/dao02313 PMID: 21553567

46. Braden LM, Sutherland BJG, Koop BF, Jones SRM. Enhanced transcriptomic responses in the Pacific salmon louse *Lepeophtheirus salmonis oncorhynchi* to the non-native Atlantic Salmon *Salmo salar* suggests increased parasite fitness. BMC Genomics. BMC Genomics; 2017; 18. https://doi.org/10.1186/s12864-017-3520-1 PMID: 28137252

47. Karlsbakk E, Sæther PA, Høstlund C, Fjellsøy KR, Nylund A. *Parvicapsula pseudobranchicola* n.sp. (Myxozoa), a myxosporidian infecting the pseudobranch of cultured Atlantic salmon (*Salmo solar*) in Norway. Bull Eur Assoc Fish Pathol. 2002;

48. Jørgensen A, Nylund A, Nikolaisen V, Alexandersen S, Karlsbakk E. Real-time PCR detection of *Parvicapsula pseudobranchicola* (Myxozoa: Myxosporea) in wild salmonids in Norway. J Fish Dis. 2011; https://doi.org/10.1111/j.1365-2761.2011.01248.x PMID: 21401644

49. Køie M, Karlsbakk E, Einen ACB, Nylund A. A parvicapsulid (Myxozoa) infecting *Sprattus sprattus* and *Clupea harengus* (Clupeidae) in the Northeast Atlantic uses Hydroides norvegicus (Serpulidae) as invertebrate host. Folia Parasitol (Praha). 2013; https://doi.org/10.14411/fp.2013.016

50. Hansen H, Poppe TT, Markussen T, Karlsbakk E. Seatrout (Salmo trutta) is a natural host for *Parvicapsula pseudobranchicola* (Myxozoa, Myxosporea), an important pathogen of farmed Atlantic salmon (Salmo salar). Parasites and Vectors. 2015; 8: 1–4. https://doi.org/10.1186/s13071-014-0608-1 PMID: 25561160

51. True K, Purcell MK, Foott JS. Development and validation of a quantitative PCR to detect Parvicapsula minibicornis and comparison to histologically ranked infection of juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), from the Klamath River, USA. J Fish Dis. 2009; 32: 183–192. https://doi.org/10.1111/j.1365-2761.2008.00975.x PMID: 19261045

52. Bradford MJ, Lovy J, Patterson DA. Infection of gill and kidney of Fraser River sockeye salmon, *Oncorhynchus nerka* (Walbaum), by *Parvicapsula minibicornis* and its effect on host physiology. J Fish Dis. 2010; 33: 769–779. https://doi.org/10.1111/j.1365-2761.2010.01178.x PMID: 20626548

53. Fujiwara M, Mohr MS, Greenberg A, Scott Foott J, Bartholomew JL. Effects of ceratomyxosis on population dynamics of Klamath fall-run Chinook salmon. Transactions of the American Fisheries Society. 2011. https://doi.org/10.1080/00028487.2011.621811

54. Ray RA, Holt RA, Bartholomew JL. Relationship Between Temperature and Ceratomyxa shasta–Induced Mortality In Klamath River Salmonids. J Parasitol. 2012; https://doi.org/10.1645/JP-GE-2737.1

55. Hemmingsen AR, Holt RA, Ewing RD, McIntyre JD. Susceptibility of Progeny from Crosses among Three Stocks of Coho Salmon to Infection by *Ceratomyxa shasta*. Trans Am Fish Soc. 1986; https://doi.org/10.1577/1548-8659(1986)115<492:SOPFCA>2.0.CO;2

56. Bartholomew JL. 3.2.4 Salmonid Ceratomyxosis [Internet]. 2012 pp. 1–9. Available: https://units.fisheries.org/fhs/wp-content/uploads/sites/30/2017/08/3.2.4-Ceratomyxosis-2014.pdf.

57. Duesund H, Nylund S, Watanabe K, Ottem KF, Nylund A. Characterization of a VHS virus genotype III isolated from rainbow trout (*Oncorhychus mykiss*) at a marine site on the west coast of Norway. Virol J. 2010; 7. https://doi.org/10.1186/1743-422X-7-19 PMID: 20102597

58. Nematollahi A, Decostere A, Pasmans F, Haesebrouck F. *Flavobacterium psychrophilum* infections in salmonid fish. J Fish Dis. 2003; 26: 563–574. https://doi.org/10.1046/j.1365-2761.2003.00488.x PMID: 14653314

59. Miller KM, Li S, Ming T, Kaukinen K, Ginther N, Patterson DA, et al. Survey of Infectious Agents Detected in Juvenile Chinook and Sockeye Salmon from British Columbia and Washington. Andrews Biol Stn. 2017; Available: http://www.npafc.org

60. Emmenegger EJ, Glenn JA, Winton JR, Batts WN, Gregg JL, Hershberger PK. Molecular identification of erythrocytic necrosis virus (ENV) from the blood of Pacific herring (*Clupea pallasii*). Vet Microbiol. Elsevier B.V.; 2014; 174: 16–26. https://doi.org/10.1016/j.vetmic.2014.08.028 PMID: 25263493

61. Hershberger P, Hart A, Gregg J, Elder N, Winton J. Dynamics of viral hemorrhagic septicemia, viral erythrocytic necrosis and ichthyophoniasis in confined juvenile Pacific herring *Clupea pallasii*. Dis Aquat Organ. 2006; 70: 201–208. https://doi.org/10.3354/dao070201 PMID: 16903231

62. Evelyn TPT, Traxler GS. Viral erythrocytic necrosis: Natural occurrence in Pacific salmon and experimental transmission. J Fish Res Board Can. 1978; 35: 903–907. https://doi.org/10.1139/f78-145

63. Eaton WD. Artificial transmission of erythrocytic necrosis virus (ENV) from Pacific herring in Alaska to chum, sockeye, and pink salmon. J Appl Ichthyol. 1990; 6: 136–141. https://doi.org/10.1111/j.1439-0426.1990.tb00572.x

64. Winton JR, Hershberger PK. 2.2.7 Viral Erythrocytic Necrosis. 2014; 1–8. Available: https://www.midcoastconservancy.org/wp-content/uploads/2018/02/Winton-Hershberger.-VEN.-2014.pdf.

65. Wessel Ø, Braaen S, Alarcon M, Haatveit H, Roos N, Markussen T, et al. Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon. PLoS One. 2017; 12: 1–24. https://doi.org/10.1371/journal.pone.0183781 PMID: 28841684

66. Takano T, Nawata A, Sakai T, Matsuyama T, Ito T, Kurita J, et al. Full-genome sequencing and confirmation of the causative agent of erythrocytic inclusion body syndrome in coho salmon identifies a new type of piscine orthoreovirus. PLoS One. 2016; https://doi.org/10.1371/journal.pone.0165424 PMID: 27788206

67. Di Cicco E, Ferguson HW, Kaukinen KH, Schulze AD, Li S, Tabata A, et al. The same strain of Piscine orthoreovirus (PRV-1) is involved in the development of different, but related, diseases in Atlantic and Pacific Salmon in British Columbia. Facets. 2018; 3: 599–641. https://doi.org/10.1139/facets-2018-0008

68. Polinski MP, Marty GD, Snyman HN, Garver KA. Piscine orthoreovirus demonstrates high infectivity but low virulence in Atlantic salmon of Pacific Canada. Sci Rep. 2019; 9: 1–22. https://doi.org/10.1038/s41598-018-37186-2

69. Garver KA, Marty GD, Cockburn SN, Richard J, Hawley LM, Müller A, et al. Piscine reovirus, but not Jaundice Syndrome, was transmissible to Chinook Salmon, *Oncorhynchus tshawytscha* (Walbaum), Sockeye Salmon, *Oncorhynchus nerka* (Walbaum), and Atlantic Salmon, Salmo salar L. J Fish Dis. 2016; 39: 117–128. https://doi.org/10.1111/jfd.12329 PMID: 25630226

70. Lapatra SE, Fryer JL, Wlngfield WH, Hedrick RP. Infectious hematopoietic necrosis virus (IHNV) in coho salmon. J Aquat Anim Health. 1989; https://doi.org/10.1577/1548-8667(1989)001<0277:IHNVII>2.3.CO;2

71. Eaton WD, Hulett J, Brunson R, True K. The first isolation in north america of infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in coho salmon from the same watershed. J Aquat Anim Health. 1991; https://doi.org/10.1021/bc200148v

72. Irvine JR, O'Neill M, Godbout L, Schnute J. Effects of smolt release timing and size on the survival of hatchery-origin coho salmon in the strait of georgia. Prog Oceanogr. Elsevier Ltd; 2013; 115: 111–118. https://doi.org/10.1016/j.pocean.2013.05.014

73. Beamish RJ, Sweeting RM, Lange KL, Neville CM. Changes in the Population Ecology of Hatchery and Wild Coho Salmon in the Strait of Georgia. Trans Am Fish Soc. 2008; https://doi.org/10.1577/T07-080.1

Viruses **2012**, *4*, 140-166; doi:10.3390/v4010140

EXHIBIT J-8

OPEN ACCESS

viruses

ISSN 1999-4915

www.mdpi.com/journal/viruses

Review

Immunity to Fish Rhabdoviruses

Maureen K. Purcell [1,*], Kerry J. Laing [2] and James R. Winton [1]

[1] Western Fisheries Research Center, United States Geological Survey, 6505 NE 65th St., Seattle, WA 98115, USA; E-Mail: jwinton@usgs.gov

[2] Department of Medicine, University of Washington, 1100 Fairview Ave. N., Seattle, WA 98109, USA; E-Mail: klaing@fhcrc.org

* Author to whom correspondence should be addressed; E-Mail: mpurcell@usgs.gov; Tel.: +1-206-526-6282 ext. 252; Fax: +1-206-526-6654.

Received: 1 December 2011; in revised form: 21 December 2011 / Accepted: 10 January 2012 / Published: 18 January 2012

Abstract: Members of the family Rhabdoviridae are single-stranded RNA viruses and globally important pathogens of wild and cultured fish and thus relatively well studied in their respective hosts or other model systems. Here, we review the protective immune mechanisms that fish mount in response to rhabdovirus infections. Teleost fish possess the principal components of innate and adaptive immunity found in other vertebrates. Neutralizing antibodies are critical for long-term protection from fish rhabdoviruses, but several studies also indicate a role for cell-mediated immunity. Survival of acute rhabdoviral infection is also dependent on innate immunity, particularly the interferon (IFN) system that is rapidly induced in response to infection. Paradoxically, rhabdoviruses are sensitive to the effects of IFN but virulent rhabdoviruses can continue to replicate owing to the abilities of the matrix (M) protein to mediate host-cell shutoff and the non-virion (NV) protein to subvert programmed cell death and suppress functional IFN. While many basic features of the fish immune response to rhabdovirus infections are becoming better understood, much less is known about how factors in the environment affect the ecology of rhabdovirus infections in natural populations of aquatic animals.

Keywords: novirhabdovirus; interferon; cell-mediated immunity; neutralizing antibody; immune evasion; non-virion; apoptosis; persistent infections; host-cell shutoff

---

1. Introduction

Rhabdoviruses are single-stranded RNA viruses and important pathogens of both wild and cultured fish throughout North America, Asia, and Europe [1]. Fish are hosts to a number of rhabdovirus species including: Infectious hematopoietic necrosis virus (IHNV), Viral hemorrhagic septicemia virus (VHSV), Hirame rhabdovirus (HIRRV), Snakehead rhabdovirus (SHRV) Spring viremia of carp virus (SVCV), Pike fry rhabdovirus (PRV), Starry flounder virus, and Ulcerative disease rhabdovirus (UDRV) [2,3]. Several of these viruses (IHNV, VHSV, and SVCV) are reportable to The World Organization for Animal Health (OIE). IHN primarily occurs in salmon and trout [4] and SVC primarily afflicts cyprinid fishes [5], while the list of VHS susceptible hosts continues to grow [6]. These viruses cause significant mortality and morbidity in both wild and cultured fish [1]. Consequently, many rhabdoviruses are well studied in their select fish hosts and the main mechanisms of protection of fish against rhabdoviruses are becoming clear. More detailed understanding of fish-rhabdovirus interactions will continue to be elucidated as our knowledge of the teleost immune system expands.

In this review, we discuss the general characteristics of fish rhabdoviruses and the factors contributing to their pathogenesis. Next, we will consider each major arm of the immune system in the context of fish rhabdoviral infection and will evaluate how rhabdoviruses evade or circumvent host defenses. Although we primarily focus on fish rhabdoviruses, at times insight gained from two closely related mammalian rhabdoviruses—Vesicular stomatitis virus (VSV) and Rabies virus (RABV)—will be drawn for comparison. Throughout the review, we will identify research gaps that, if answered, would enable a more comprehensive understanding of immunity to fish rhabdoviruses.

2. The Fish Rhabdoviruses

2.1. Taxonomy

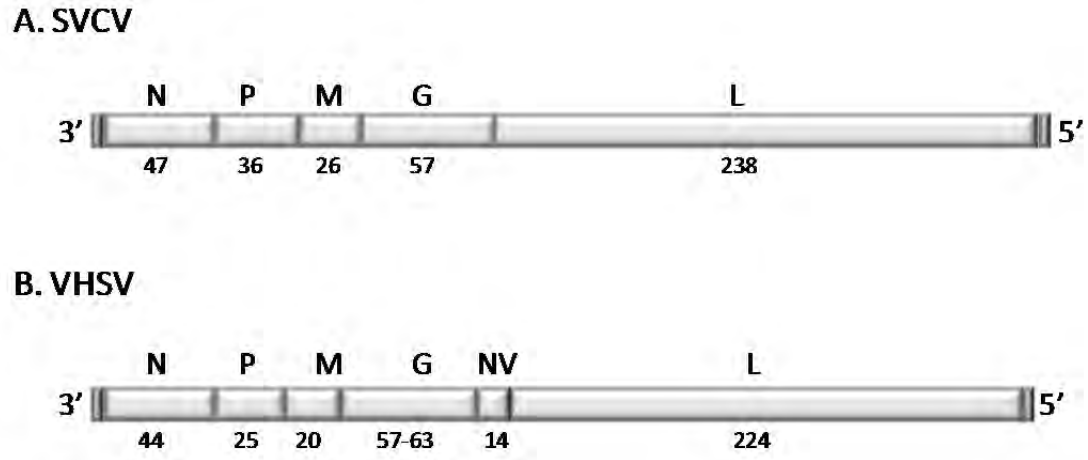
The rhabdovirus family contains viruses that are related in terms of both morphological and genetic characteristics [7]. Collectively, this growing viral family can infect a vast array of hosts in both plant and animal phyla and comprises over 160 species classified among six genera (*Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus*) [7]. The best studied of all fish rhabdoviruses are IHNV, VHSV, HIRRV and SHRV, which are accepted as members of the genus *Novirhabdovirus* [2]. SVCV is currently classified within the genus *Vesiculovirus* (typified by VSV) [2], but this taxonomic grouping is likely to change.

2.2. Structure

Rhabdoviruses are bullet-shaped, enveloped viruses with a simple negative-sense, single-stranded RNA (ssRNA) genome [8]. The typical rhabdoviral genome encodes five basic structural proteins, including the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the large polymerase (L) protein (Figure 1A) [9]. Additionally, all rhabdoviruses possess non-coding 3' leader and 5' trailer sequences. Members of the genus *Novirhabdovirus* are distinguished by the

presence a sixth gene located in the genome between the G and the L genes (Figure 1B) encoding a non-structural ('non-virion' or NV) protein [10,11] that has a role in pathogenesis as discussed below.

Figure 1. Genome organization and predicted molecular weights (MW) in kilodaltons of (**A**) a typical member of the genus *Vesiculovirus*, spring viremia of carp virus (SVCV) and (**B**) a typical member of the genus *Novirhabdovirus*, Viral hemorrhagic septicemia virus (VHSV). Predicted SVCV MW are based on [9] and for VHSV on [11].



2.3. Infection Cycle

Understanding the viral infection cycle is important to define where the host immune system encounters virus-associated molecules. The general rhabdovirus life cycle, derived from VSV, has been reviewed extensively elsewhere and involves the five structural proteins found in all rhabdoviruses [8]. Briefly, the rhabdovirus enters its target cell by receptor-mediated endocytosis, which is triggered following engagement of a cell surface receptor by the viral G protein. The viral and endosomal membranes subsequently fuse and the viral nucleocapsid is released into the cytoplasm of the host cell. Within the cytoplasm, the viral genes are sequentially transcribed from the genome using an RNA-directed RNA polymerase that accompanies the infecting virion, viral proteins are synthesized by host cell machinery, and new copies of the genome are synthesized from a full-length, single-stranded RNA anti-genome. To package the new virions, the N, L and P proteins, synthesized by free ribosomes in the cell cytoplasm, bind to the newly synthesized copies of the viral RNA genome to form the ribonucleoprotein (RNP) core, which associates with the M protein to produce the RNP-M complex. The G protein is synthesized by endoplasmic reticulum (ER)-bound ribosomes. It is glycosylated and further modified within the ER and Golgi apparatus prior to transport and insertion into the plasma membranes on the host cell surface. The RNP-M complex migrates to regions of the plasma membrane enriched with viral G proteins. The G protein studded host cell plasma membrane is subsequently captured by RNP-M protein complex as it buds from the cell to create fully enveloped rhabdovirus.

2.4. Disease Manifestations

Viruses generally follow one of two strategies to ensure survival and transmission: the 'hit and run' or the 'hit and stay' [12]. As acute cytolytic viruses that cause high mortality, fish rhabdoviruses are typically considered 'hit and run' viruses. Their associated diseases are commonly characterized as acute hemorrhagic septicemias that impact multiple organs and present common clinical signs such as skin darkening, ascites, and exopthalmia [1]. However, varied forms of disease have been described for both IHN (hematopoietic and neurotropic) and VHS (acute, chronic and nervous) in rainbow trout (*Oncorhynchus mykiss*) [13,14], and a persistent infection/asymptomatic carrier state for both VHSV and IHNV has been reported that is often, but not always, associated with neural tissues [15–19]. There has been relatively little study of these alternative disease manifestations since most laboratory studies employ acute challenge models with virus doses sufficient to give reproducibly high mortality.

2.5. Vaccination Strategies

In general, survivors of acute fish rhabdoviral infections develop long-lasting immunity, which is broadly protective against multiple virus strains of the same species [20,21]. In mammals, long-lasting immunity and anamnesis are characteristics of the adaptive immune response whereby memory T and B lymphocytes are developed during the initial infection by a pathogen. These cells then maintain surveillance for that same pathogen, perhaps for the life of the host, and are able to respond rapidly in the event of subsequent infection [22]. Vaccines exploit this characteristic by eliciting an anamnestic response prior to initial exposure to a pathogen, thus protecting the host against future infection. A variety of approaches have been investigated for vaccinating fish against rhabdoviral infections including attenuated strains, inactivated virus, subunit vaccines, and DNA vaccination [23–26]. These approaches are all efficacious to a degree (reviewed by [23,24,27]) but—with the exception of the IHNV DNA vaccine in Canada [28]—have not been licensed due to concerns regarding safety, consistency or cost. To overcome some of the safety concerns associated with DNA vaccines (see [29]), new vector constructs that use fish inducible promoters and a suicide gene (the IHNV M protein) to limit long-term survival of vaccine DNA in the muscle tissues have been developed and show promise [30,31].

Experimental DNA vaccines based on the rhabdoviral G protein of IHNV, VHSV, HIRRV and SVCV have shown efficacy in a number of different fish host species [32–36]. These G protein vaccines have been widely used as tools to probe the mechanisms of protective immunity in fish (for review see [37,38]). They elicit an early, nonspecific immune response that cross-protects against other viruses but not bacteria, while specific immunity arises later [39–41]. The G protein is the protective antigen but only in its correct conformation (for review see [23,24]). Large-scale production of glycosylated and folded G proteins has been difficult, limiting their commercial potential as subunit vaccines but recent successes with insect-based systems may change this [42]. However, the major barrier that remains to commercial rhabdoviral vaccination is the ability to mass vaccinate a large number of highly susceptible small fish. All fish rhabdoviral vaccines developed to date require individual handling and are typically administered via intramuscular or intraperitoneal injection. Novel

immersion or oral vaccination approaches (*i.e.*, [43]) may eventually provide a solution to this barrier (reviewed recently in [44]).

3. Immune Response

3.1. Mammalian Paradigm of Anti-Rhabdoviral Immunity

Conserved pattern recognition receptors (PRRs) of the innate immune system, such as the Toll-like receptor (TLR) and RIG-like receptor (RLR) families, detect 'pathogen associated molecular patterns' (PAMPs) and signal through conserved pathways to activate innate immune effector molecules [45]. In general, induction of anti-viral immunity follows recognition of viral nucleic acids by TLRs 3, 7 and 8 or the RLRs retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) and viral glycoproteins by TLRs [46]. This paradigm holds true for rhabdoviruses. For instance, engagement of both TLR7 by ssRNA and TLR4 by the viral G protein is required to mount an effective immune response against VSV in mice [47–49]. Both RIG-I and MDA5 are required for immunity against RABV [50] while RIG-I is also implicated in sensing VSV [51].

Signaling through PRRs leads to the activation of immune effector molecules, particularly the interferon (IFN) system. The IFNs mediate the primary early response to viruses. IFNs are powerful set of pleiotropic cytokines with diverse roles in regulating the immune system and inducing an anti-viral state. They are divided into three major classes of gene families in mammals: Type I (IFN-α, β, ε, κ, τ and ω), Type II (IFN-γ) and Type III (IFN-λ) [52,53]. Type I IFNs are produced by a variety of cell types while Type II IFN expression is restricted to immune cells. It is well-established that RABV and VSV cannot replicate in type I IFN-alerted cells indicating the importance of type I IFN activity in controlling rhabdoviruses (reviewed in [54]). Furthermore, IFN-γ has been shown to reduce infection of murine neurons and neuroblastoma cells by VSV, whereby neuronal nitric oxide synthase (nNOS) activity is enhanced by IFN-γ to elicit viral control via production of reactive nitric oxide [55,56].

The IFNs signal through the conserved JAK/STAT pathway and up-regulate the expression of >300 IFN stimulated genes (ISGs) [57,58], which establish the 'anti-viral' state [59]. Many of these ISGs have direct anti-viral function (*i.e.*, Mx [60]) while the functions of other ISGs remain unknown. The ISG protein kinase R (PKR) binds double-stranded RNA (dsRNA) to inhibit viral protein synthesis [61]. Furthermore, mice deficient in PKR are more susceptible to VSV infections confirming the importance of this PRR in controlling rhabdoviruses [61].

The adaptive immune system is also an important component of the anti-rhabdoviral immunity. This arm of the immune system employs two classes of lymphocytes—B cell and T cells—that very specifically recognize viral antigens. B cells are absolutely essential for survival to VSV infection in mice [62]. They secrete neutralizing antibodies directed against the viral G protein, and are highly protective against VSV. Due to the repetitive, polyvalent nature of G protein epitopes on VSV virions—a typical characteristic of T cell independent antigens—these neutralizing antibodies are initially (days 2–6) produced in a T cell independent manner and represent predominantly the IgM isotype. Their secretion later becomes T cell dependent generating long-lived antibodies from both the IgM and IgG isotypes [63]. In mammals, there are two major T cell subsets: Cytotoxic T lymphocytes

(CTL) that typically utilize the CD8 co-receptor and helper T cells (T_H) that utilize the CD4 co-receptor [22]. Studies in immune deficient mice highlight the requirement of CD4+ T cells for an optimal immune response against VSV and show that while CD4+ T cells can compensate for the their absence, CD8+ T cells also have anti-VSV activity [62]. In mammals, CTLs are responsible for specific cell-mediated cytotoxicity (CMC) while natural killer (NK) cells of the innate immune system are the primary effectors of nonspecific CMC [22]. Mammalian NK cells participate in the early innate antiviral immune response through non-antigen specific CMC of infected targets and secretion of IFN-γ, while the CTL response occurs at later time point, and is MHC class I-restricted and antigen-specific.

3.2. Viral Recognition in Fish

Pattern recognition of rhabdoviruses in fish is likely to mirror that observed in mammals [64]. Although there is limited functional characterization of receptor binding activity and signaling, teleost fish are known to possess the major TLR families plus an expanded set of unique non-mammalian TLR genes and gene variants (reviewed by [65,66]), as well as genes encoding RIG-I, MDA5, laboratory of genetics and physiology 2 (LGP2) and the associated signaling molecule mitochondrial antiviral signaling (MAVS) protein [67–69]. Fish possess orthologs to typical anti-viral TLRs including TLR3, TLR7 and TLR8 [70–72], in addition to novel TLRs (TLR22) that appear to bind different forms of RNA [73], suggesting there is conservation in recognition of viral nucleic acids. In contrast, most fish species either lack TLR4 or possess highly divergent TLR4-like genes relative to mammalian TLR4 (e.g., zebrafish (*Danio rerio*)) [74]. Thus, the phenomenon of viral glycoprotein recognition ascribed for TLR4 in mammals is unlikely to be conserved in fish species. However, the G proteins of novirhabdoviruses are known to trigger the synthesis of IFN in rainbow trout cells suggesting that capacity of the innate immune system to recognize rhabdoviral glycoproteins is conserved [75,76]; although the PRR(s) involved in this interaction are currently undetermined. The IFN response to the G protein can be triggered in both immune [75] and non-immune cells [76] and IFN induction is dependent on a peptide region that contains a canonical integrin binding site [77]. Other PRRs such as RIG-I, MDA5 and LGP2 (and their associated adaptor MAVS) activate the IFN system when overexpressed in fish cells, which correlates with protection against rhabdoviral infection [69,78].

3.3. Innate Immune Response to Fish Rhabdoviruses

Characterization of the innate immune response to fish rhabdoviruses has accelerated due to the genomic information available for model and commercially-important fish species. Genes encoding critical cytokines, chemokines, and other innate effectors have been identified in fish including those encoding both type I IFNs (also called IFNφ) and type II IFNs (also called IFNγ) [79–89]. Fish type I IFNs (IFNφ) are split into two major groups, which differ in the number of conserved cysteine (C) residues (group I—2C and group II—4C) [83,84] and appear to bind different receptors [90,91]. In general, group I IFNs are expressed in a wide range of tissue types while group II IFNs are more abundant in hematopoietic-derived tissues and cells [84]. Multiple gene copies of IFNγ exist in fish species but these genes are not duplicated to the same extent as the type I IFNs (reviewed in [92]). The

phylogenetic relationships among fish IFNs and the associated nomenclature are confusing and disputed; the review by Zou and Secombes [92] provides a useful table summarizing the fish IFNs identified to date.

Like their mammalian counterparts, replication of fish rhabdoviruses is inhibited by pre-activation of the IFN system regardless of the method used, including using IFN-containing supernatants [93,94], poly I:C [95], rhabdoviral G protein [39,40,75] or recombinant IFN [96,97]. Prior to the identification of the IFN genes themselves, activation of a functional IFN response was inferred by virus plaque reduction [98], transcription of the Mx gene or other ISGs (*i.e.*, [99–101] and many other studies), or activation of an Mx promoter-reporter assay [102]. Early studies clearly indicate functional IFN in sera derived from fish infected with VHSV [93] , IHNV [98] or SVCV [94]. Later studies confirmed these results and demonstrate transcriptional up-regulation of both group I and II IFN genes after infection with these same viruses [84,85,103]. IFNγ genes are also regulated early in rhabdoviral infection [85].

Rhabdoviral infections are controlled by fish group I and II IFNs. Recombinant group I IFNs inhibit rhabdoviral replication [84,96,97,103,104] while the effects of group II IFNs on rhabdoviruses vary (e.g., [84]). A recent study demonstrates that recombinant group II IFNs protect zebrafish against SVCV infection and induce a rapid and transient up-regulation of ISGs whereas those from group I up-regulate both ISGs and pro-inflammatory cytokines in a more sustained fashion [103]. Similarly, fish IFNγ also elicits an immune response related to controlling viruses. Recombinant IFNγ up-regulates a number of ISGs [105] and displays anti-viral activity [106] in a variety of Atlantic salmon (*Salmo salar*) cell-lines. At least some of the anti-viral activity ascribed to IFNγ may be due to the ability of IFNγ to increase transcription of group I IFNs [106]. However, recombinant IFNγ does not improve survival of zebrafish infected with SVCV [103], questioning whether rhabdoviruses are directly controlled via the activity of this cytokine.

Rhabdoviral infection and/or G protein vaccination induce a characteristic transcriptional pattern dominated by ISGs that are conserved throughout vertebrates, as well as the expression of novel antiviral genes (for a comprehensive review see [107]). Vaccination with the rhabdoviral G protein primes the systemic up-regulation of these ISGs [108–110] and the timing of this response correlates with nonspecific protection from virulent IHNV and VHSV challenge [39,40]. Fish rhabdoviral infection also result in a rapid IFN and ISG response—similar to that observed early after G protein vaccination—which correlates with virus levels in the tissues, but does not necessarily correlate with protection [101,111–113]. This paradox is suggestive of a virus-host race in which rapid replication of the fish rhabdoviruses competes kinetically against mobilization of innate immune mediators. Rapid replication is a hallmark of highly virulent IHNV strains [114–116]. Rainbow trout progeny groups with greater genetic resistance to IHNV have lower *in vivo* viral replication starting at less than 24 h post-infection [117], which is a relatively limited time frame for the host to induce a response that relies on new protein synthesis (*i.e.*, the IFN system). This suggests that constitutive or other innate barriers to viral replication are also important for overall disease resistance. Other studies have found that VHSV resistance in trout is correlated with *ex vivo* replication of VHSV in fin tissues, implicating epidermal tissues as pivotal in the anti-VHS defense system [118–120]. Host-virus dynamics at virus entry points (*i.e.*, the fins [121]) may help limit the internal spread of viral infection by alerting systemic sites via IFNs and other cytokines.

3.4. Humoral Immune Response to Fish Rhabdoviruses

Neutralizing antibodies induced by infection and/or vaccination are critical components of long-term adaptive immunity to fish rhabdoviruses (reviewed in [122]). Passive immunization with sera containing neutralizing antibodies protects recipient juvenile trout from IHN and Pacific herring from VHS, even when titers fall below detectable levels [40,123–125]. Most studies of fish rhabdoviruses have focused on the highly protective neutralizing antibody response but it is possible that non-neutralizing or other types of antibodies may also play a role. *In vitro* rhabdoviral neutralization is complement-dependent but the exact mechanism by which complement aids viral neutralization is still unclear [126]. Neutralizing antibodies are unlikely to play a role in surviving the acute infection phase in coldwater fish species since neutralizing antibodies typically are not detectable until several weeks post-infection. Study of attenuated or low virulence virus types indicates that a certain threshold of virus replication must occur for fish to develop a broadly protective antibody response, even if a robust innate response is induced [113,127].

Lorenzen and LaPatra [122] published a comprehensive review of the antibody response to fish rhabdoviruses in 1999, and since then, this area has not received much new attention. Certainly, studies of the G protein DNA vaccines over the past decade have reaffirmed the central role of neutralizing antibodies in protective immunity [33,34,128–133]. However, genomic approaches combined with functional studies have brought exciting new insights into B cell biology in teleost fish. Fish B cells show phagocytic behavior suggesting that fish B cells may also function as part of the innate immune system [134]. Furthermore, it is now known that teleost fish possess three or more immunoglobulin isotypes including IgM, IgD and IgT (IgT is also called IgZ in zebrafish) [135–137]. A recent study indicates that B cells expressing IgM respond to antigenic stimulation in systemic tissues while B cells expressing IgT are key to the mucosal immune response [138]. To date, there has been no characterization of IgT during fish rhabdoviral infections. However, tools such as monoclonal antibodies to fish IgM and IgT now exist [138,139], which will allow the characterization of mucosal immunity and its relationship to systemic protection against rhabdoviruses. Finally, there has been much progress in unraveling the complexity of the fish complement system [140], which may help to finally define which complement components contribute to virus neutralization.

3.5. Cellular Immune Response to Fish Rhabdoviruses

Although DNA vaccination with the novirhabdoviral G protein triggers production of protective neutralizing (and perhaps other) antibodies, high levels of specific protection after DNA vaccination are also observed without detectable neutralizing antibodies [33,130] indicating a potential role for specific cellular immunity. Major advances have been made recently in our understanding of fish T cells (as reviewed in [141]). Teleost fish possess a wide range of T cell associated genes, including genes that encode T cell receptor chains and various other T cell associated co-receptors, co-stimulatory molecules and cytokines [141]. Many of these genes show up-regulated expression following rhabdoviral infection and/or G protein DNA vaccination [110,142]. There is a limited understanding of the role T cells play during fish rhabdoviral infection with most work focusing on cell-mediated cytotoxicity (CMC) (discussed below). However, VHSV infection and DNA vaccination with the

VHSV G protein induces clonal expansion of T cells, as shown by spectratype analysis of the complementarity-determining region 3 (CDR3) of the TCR-β chain [143,144], supporting a role of teleost T cells in controlling this virus. Interestingly, the dominant CDR3 profiles are the same for both VHSV infection and G protein DNA vaccination suggesting that important T cell epitopes are localized within the G protein. Future studies examining the role of T cells during rhabdoviral infection will benefit from new reagents that can discriminate among T cell subsets (reviewed in [141]), including monoclonal antibodies against the CTL surface marker CD8 [145].

Studies using rainbow trout, channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*) and crucian carp (*Carassius carassius*) provide evidence for both specific and nonspecific CMC in fish (reviewed in [146,147]). Two different cell types are responsible for nonspecific CMC in channel catfish, the nonspecific cytotoxic cells (NCC) and NK-like cells [148–150]. Common carp neutrophils also possess nonspecific CMC activity, evidenced by spontaneous killing of human tumor cell lines [151]. Studies of mammals and other vertebrate species indicate that NK cell activity is mediated by many different receptor types that can be specific to an individual animal species or even to a cell lineage [152]. Candidate NK receptor families in fish include the novel immune type receptors (NITRs), novel Ig-like transcripts (NILTs), leukocyte immune type receptors (LITRs), and possibly others (reviewed in [152]). Genomic characterization of fish species has enabled the identification of these polymorphic and polygenic receptor families, but there has been limited validation that these receptors have a functional role in NK activity.

Functional studies of specific CMC were made possible in fish following the development of isogenic fish and major histocompatibility complex (MHC) I matched cell lines for both crucian carp and rainbow trout [153–155]. Somamoto *et al.* [155] demonstrated that peripheral blood and kidney derived lymphocytes from crucian carp exhibit a specific CMC response to MHC-matched cells infected with Crucian carp hematopoietic necrosis virus (CHNV; an uncharacterized fish rhabdovirus). Similarly, peripheral blood lymphocytes from rainbow trout infected with VHSV exhibit significant CMC to MHC class I matched target cells infected with VHSV but not to VHSV infected xenogeneic targets [154]. However, rainbow trout survivors re-exposed to VHSV mounted a kinetically faster, VHSV-specific CMC response, as well as a nonspecific CMC response against VHSV infected xenogeneic targets (starting at 11 days post-infection). The delayed nonspecific CMC response in trout contradicts the mammalian paradigm where primary CMC is nonspecific and results from NK cell activity while later CMC is pathogen-specific due to clonal expansion and action of virus specific CTLs [22]. Taken together, these studies suggest a role for cellular immunity—particularly CMC—in the host response to fish rhabdoviruses. However, more basic research is needed to definitely identify the cells that contribute to key cellular effector functions in fish.

3.6. Modulation of Immunity by Environmental Parameters

For poikilothermic vertebrates, temperature is a critical factor in host-pathogen interactions because most aspects of the host's physiology, including the strength and speed of the immune response, as well as the replication rate of the pathogen are temperature dependent [156]. Among fish rhabdoviruses, it is well known that the severity of VHS and SVC alters with changing temperatures in the spring and fall [157,158]. However, while the *in vitro* replication rate of fish rhabdoviruses

increases with temperature to a maximum upper limit [158], the severity of disease is generally less at higher temperatures which may be due to the greater efficiency of the fish immune response [159,160]. As such, modulation of temperature has been explored as a basis for protection or vaccination of fish against rhabdovirus infections [161,162].

At optimal temperatures, fish surviving rhabdovirus infections develop a robust immune response and clear the virus below detectable levels [163]. In contrast, fish held at lower temperatures may rely more on innate immunity and have inhibited or delayed specific immune responses [156,164,165]. Low or cold water temperatures are also linked to development of persistent rhabdoviral infections [15,17]. For example, rainbow trout held at 4 °C had detectable VHSV in the brain for over 400 days post-infection and displayed no clinical signs of VHS or detectable serum neutralizing antibody titers [17]. These studies support the hypothesis that rhabdoviral persistence is related to suppression of the adaptive immune response. However, it is not clear whether individuals become long-term carriers or if the virus persists by cycling in the population among naïve and/or convalescent hosts [18]. If individuals are truly persistently infected, it raises interesting questions of how normally acute cytolytic viruses modulate replication and limit cytopathology.

Other environmental factors may also affect disease resistance in fish. Intriguingly, the CMC response in fish immunized with the N protein of VHSV was suppressed during winter months despite constant water temperature and light regimen; this result may indicate that endogenous biological variables (e.g., reproductive cycle) or other seasonal factors influence anti-viral immunity [166]. Resistance to fish rhabdovirus infections has also been shown to be modulated by diet [167,168]. Thus, in addition to temperature, other environmental factors such as age, diet, seasonality and reproductive status will be important areas for study in order to fully understand the immune response to fish rhabdoviruses.

4. Rhabdoviral Immune Evasion Mechanisms

4.1. Overview

A pathogenic virus must possess some mechanism to evade or limit innate host defenses, disrupt normal cellular processes, and gain preferential transcription and translation of its own genes [169]. Viruses have evolved many strategies to overcome host defenses. In response, the vertebrate immune system continuously evolves counter-strategies against the virus. This co-evolutionary arms race has led to great diversity in the host-virus relationship as viruses evolve new strategies to limit, evade or otherwise subvert the immune system. Here we will outline important aspects of immune evasion by mammalian rhabdoviruses. Next, we will consider evidence indicating a critical role for the fish rhabdoviral M and NV proteins in immune evasion.

4.2. Mammalian Rhabdoviral Immune Evasion

As mentioned above, rhabdoviruses cannot replicate in IFN-primed cells (reviewed in [54]). However, the rapid and strong induction of the host IFN system following detection of invading rhabdoviruses is not sufficient to prevent the replication of virulent rhabdoviruses. Rhabdoviruses are thought to directly interfere with critical immune effector functions including the interferon system to

evade immune control. They may also interfere with programmed cell death processes (apoptosis) and mediate global inhibition of host cellular gene expression and thereby inhibit transcription-dependent host cell defenses, a phenomenon known as host-cell shutoff [170]. RABV and VSV use very different strategies to disrupt mammalian host defenses. RABV relies on the P protein to limit IFN production, antagonize IFN signaling and directly interfere with IFN-induced antiviral molecules [171,172]. In contrast, VSV relies on the M protein to facilitate host-cell shutoff [170]. This mechanism limits RNA transcription by suppressing all host RNA polymerases [170], inhibits nucleocytoplasmic transport by blocking nuclear pore components [173], and prevents translation of host genes into proteins [174]. Shutoff of host cellular processes likely increases the resources available for viral transcription and translation, while decreasing production of anti-viral host proteins.

Rhabdoviral infection induces apoptosis in infected mammalian cells (reviewed in [169]). Apoptosis is a type of programmed cell death that creates characteristic morphological and biochemical changes and is mediated by proteolytic enzymes called caspases [175]. In infection-associated apoptosis, there arises a common question concerning whether the cell death is due to viral factors or to the host antiviral response. Both factors may be responsible. For instance, reverse genetic analysis of VSV revealed that apoptosis occurs by two distinct pathways; one pathway is associated with the M protein while the other pathway relies on host gene expression [176,177]. The M protein-mediated apoptosis may be a byproduct of the host-cell shutoff activity, since the suppression of host cellular gene expression and translation is most likely incompatible with long term cell survival. This hypothesis is supported by the finding that host-cell shutoff and apoptosis are genetically correlated and involve the mitochondrial (intrinsic) pathway of apoptosis and caspase 9 [176,177]. The second pathway is hypothesized to result from a typical host anti-viral response and requires both new host gene expression and caspase 8 to occur [176,178]. However, rapid induction of apoptosis is not effective at inhibiting VSV replication in most cell types suggesting that this is not necessarily an effective anti-viral response [177,179].

Antigenic/epitope variation is another strategy used by certain viruses, bacteria and parasites to evade the immune system and escape from protective humoral and/or cellular immune responses. Among viruses, this strategy is best known as an important feature in the biology of Influenza virus or Human immunodeficiency virus [180]. For rhabdoviruses, evidence for immune selection by antibody escape has been shown in variant laboratory strains of both RABV and VSV whereby escape mutants lose detectable reactivity with monoclonal antibodies specific for neutralizing epitopes on the virus G protein [181,182]. Field isolates of VSV from endemic areas show some evidence of immune selection [183,184]. Nevertheless, while playing a role in virus evolution, this strategy does not appear to be a critical factor in the persistence or epidemiology of rhabdoviruses in that antigenic strains of RABV and VSV continue to circulate in endemic areas without sequential replacement. Moreover, immunity conferred by infection or vaccination with one strain is broadly protective and likely due to conservation of some neutralizing epitopes on the G proteins of rhabdoviruses [185].

4.3. Fish Rhabdoviral Immune Evasion: Role for M Protein

Showing similarity to VSV, the fish novirhabdovirus IHNV possesses host-cell shutoff ability that is specifically mediated by the M protein [186]. Transfection of cultured Chinook salmon embryonic

cells (CHSE-214) with the IHNV M protein also induces host morphological changes consistent with apoptosis [186], indicating cell death can occur in the absence of live virus. In contrast, there is no observable molecular or cellular effect following transfection of the IHNV P protein [186]. To date, host cell-shutoff has only been documented for IHNV and not for the other fish rhabdoviruses. The availability of reverse genetic tools for IHNV, VHSV and SHRV [187–189] would allow further dissection of this response in fish, such as the development of M protein mutants deficient in host-cell shut off activity.

4.4. Fish Rhabdoviral Immune Evasion: Role for NV Protein

Attempts to identify a function for the NV protein have led to contradictory results. Overexpressed IHNV NV causes cell rounding in CHSE-214 cells but not apoptosis leading to speculation that NV interacts with host cytoskeletal elements [186]. SHRV NV is dispensable for efficient replication in cultured fathead minnow cells (EPC cell line; [190,191]) and *in vivo* virulence in zebrafish [188,192]. In contrast, the IHNV NV is essential for optimal growth in EPC cells and virulence in rainbow trout [193]; a similar observation was made using a VHSV NV knockout in the yellow perch (*Perca flavescens*) host [194]. Two new studies now indicate a role for NV in delaying apoptosis and suppressing the host IFN system [187,195].

Many viruses possess strategies to delay or prevent apoptosis to prolong viral replication. Recently, Ammayappan and Vakharia [187] demonstrated that the VHSV NV protein has the ability to delay the onset of apoptosis in cell culture. In their study, recombinant VHSV lacking NV induced apoptosis sooner than wild-type VHSV. The anti-apoptotic function could be recovered by the IHNV NV [187], suggesting the NV protein's anti-apoptotic function may be conserved despite low amino acid similarity between the VHSV and IHNV NV proteins (~48%) [193]. Furthermore, VHSV infection induces the activity of the initiator caspases 3, 8, and 9 [187] implicating the involvement of multiple apoptotic pathways that may be induced by specific viral components (e.g., M protein) and/or by host components (e.g., mediated by IFN, PKR *etc.*). More studies are needed to determine which apoptotic pathway(s) are being targeted for delay by the VHSV NV protein.

A second recent study indicates that the NV also functions to limit the host IFN response in fish. Choi *et al.* [195] demonstrated that recombinant IHNV lacking the NV gene induced greater transcription of the rainbow trout Mx and IFN1 in RTG-2 cells and had higher levels of functional IFN, as measured by an Mx reporter assay [102]. The study demonstrated that the NV protein is actively transported to the nucleus and that nuclear transport and anti-IFN activity were dependent on a unique nuclear localization signal (NLS). Although the NLS was conserved among all IHNV strains examined, it was not conserved in VHSV indicating that further study is needed to determine if the NV from all novirhabdoviruses function in a similar manner. Furthermore, it would be interesting to determine if the anti-apoptotic activity of the NV protein (described above for VHSV) is genetically correlated with the ability to limit IFN and is dependent on nuclear localization.

4.5. Limited Evidence for Antigenic Escape

Analogous to RABV and VSV, isolates of fish rhabdoviruses show a level of antigenic diversity [4,5,157,196], and respond to immune selection with monoclonal antibodies by generating

escape mutants [197]. Analysis of the amino acid sequences encoded by the G protein gene of a diverse set of field isolates of IHNV showed that the maximum diversity occurred in the central region of the protein that contained many of the antigenic determinants [198] suggesting that immune selection may be helping to drive virus evolution. This central region of the G gene was used by Kurath *et al.* [199] to show that, compared to stocks of wild anadromous salmonids in their native range, genetic diversity was highest among trout populations reared in aquaculture where a high rate of virus replication/infection and the presence of large numbers of recovering/recovered animals would be expected to drive a greater level of immune selection. Comparison of the nonsynonymous and synonymous substitutions present in the G gene sequences obtained from cultured rainbow trout IHNV isolates suggests the possibility that some specific amino acids may be under positive selection [200]. Furthermore, this phylogenetic analysis revealed that isolates of IHNV from trout aquaculture appeared to be undergoing a higher rate of evolution with the most recent isolates positioned at branch tips, unlike isolates from wild populations [199]. However, within the range of genetic strains or serological variants that comprise a given species of fish rhabdovirus, laboratory studies show broad protective immunity in fish that recover from infection with a given strain of the virus or are vaccinated using antigens from a specific strain [20,21]. Thus, while these data indicate that immune selection may be an important driver of genetic diversity and virus evolution, fish rhabdoviruses appear to be incapable of undergoing complete antigenic escape, perhaps due to the presence of a functionally essential, and thus highly conserved, regions of the G protein. However, more study is needed of the immunological responses to alternate disease manifestations, such as persistent infections, to determine how the virus evades the host antibody response.

5. Future Directions

Interactions between the immune system and rhabdoviruses are well studied for certain fish rhabdoviruses in certain hosts (*i.e.*, IHNV and VHSV in salmonid species), but there are still gaps in our knowledge. Over the past decade, much advancement has been made in understanding the genomic architecture of the fish immune system, which will facilitate future functional studies to fill in these gaps. There has also been progress in identifying the viral determinants of immune evasion for VHSV and IHNV using reverse genetics, but it is not known if these strategies are broadly conserved among all members of the genus *Novirhabdovirus.* Additionally, more work is needed to better define the detailed mechanisms underlying these evasion strategies. The knowledge gaps are even greater for the SVCV and other vesiculo-like viruses. It is likely that the M protein-mediated host-cell shutoff is conserved in SVCV, but other novel mechanisms may also exist. For probing some of these questions, zebrafish may serve as an excellent model because many tools are available to genetically manipulate this fish species. However, there should be some caution with relying exclusively on model fish species as some aspects of the host-pathogen relationship may not be represented during infections of non-natural hosts.

How highly virulent fish rhabdoviruses persist in populations is a long unanswered question. The answer will likely vary depending on many factors, including the rhabdoviral species, host species, host life history, types of vectors, environment, and degree of anthropogenic manipulation. However, understanding how fish rhabdoviruses interact with the host immune system can provide a new

perspective on this question. Infection with fish rhabdoviruses is often acute and associated with high mortality; survivors typically clear the virus and develop broadly protective immunity to re-infection. However, if this scenario were always to occur, a reduction in the number of susceptible hosts through mortality and increased herd immunity among recovered animals should theoretically drive the virus out of any natural population. There are several alternatives to this scenario that include, but are not limited to: (1) 'immune' fish can replicate the virus to sufficient levels for viral shedding and transmission to occur, (2) endogenous or exogenous parameters suppress fish immunity such that survivors can be re-infected, (3) fish rhabdoviruses have a broad enough host range that susceptible hosts are always available, or (4) these normally acute viruses can also persistently infect a small number of individuals to ensure survival when there are no susceptible hosts. Although there are a number of studies indicating that fish rhabdoviruses can establish persistent infection (described earlier), there remains a possibility that the perceived persistence in these studies reflects infection cycling among previously exposed individuals or in non-natural conditions. Distinguishing among these hypothesized scenarios will require the merging of immunology and epidemiology, which may be the key to unraveling the disease ecology of fish rhabdoviruses.

Acknowledgments

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Interior or the U.S. Geological Survey of any product or service to the exclusion of others that may be suitable. The authors thank Paul Hershberger, Gael Kurath and John D. Hansen for critical reviews of this manuscript.

Conflict of Interest

The authors declare no conflict of interest.

References and Notes

1. Kurath, G.; Winton, J. Fish Rhabdoviruses. In *Encyclopedia of Virology*, 3rd ed.; Mahy, B.W.J., van Regenmortel, M.H.V., Eds.; Academic Press: Oxford, UK, 2008; pp. 221–227.
2. Whitfield, A.E.; Calisher, C.H.; Stone, D.M.; Kurath, G.; Kuzmin, I.V.; Rodriguez, L.L.; Tordo, N.; Walker, P.J.; Dietzgen, R.G.; Tesh, R.B.; *et al.* Rhabdoviridae. In *Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses*; King, M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., Eds.; Elsevier: Oxford, UK, 2011; pp. 686–714.
3. Mork, C.; Hershberger, P.; Kocan, R.; Batts, W.; Winton, J. Isolation and characterization of a rhabdovirus from starry flounder (*Platichthys stellatus*) collected from the northern portion of Puget Sound, Washington, USA. *J. Gen. Virol.* **2004**, *85*, 495–505.
4. Bootland, L.M.; Leong, J.C. Infectious haematopoietic necrosis virus. In *Fish Diseases and Disorders*; Woo, P.T.K., Bruno, D.W., Eds.; CAB International: Wallingford, UK, 1999; Volume 3, pp. 57–121.

5. Ahne, W.; Bjorklund, H.V.; Essbauer, S.; Fijan, N.; Kurath, G.; Winton, J.R. Spring viremia of carp (SVC). *Dis. Aquat. Organ.* **2002**, *52*, 261–272.
6. Al-Hussinee, L.; Lord, S.; Stevenson, R.M.; Casey, R.N.; Groocock, G.H.; Britt, K.L.; Kohler, K.H.; Wooster, G.A.; Getchell, R.G.; Bowser, P.R.; *et al.* Immunohistochemistry and pathology of multiple Great Lakes fish from mortality events associated with viral hemorrhagic septicemia virus type IVb. *Dis. Aquat. Organ.* **2011**, *93*, 117–127.
7. Fu, Z.F. Genetic comparison of the rhabdoviruses from animals and plants. *Curr. Top. Microbiol. Immunol.* **2005**, *292*, 1–24.
8. Wagner, R.R. Rhabdovirus biology and infection: An overview. In *The Rhabdovirus*; Wagner, R.R., Ed.; Plenum Press: New York, NY, USA, 1987; pp. 9–61.
9. Hoffmann, B.; Schutze, H.; Mettenleiter, T.C. Determination of the complete genomic sequence and analysis of the gene products of the virus of Spring Viremia of Carp, a fish rhabdovirus. *Virus Res.* **2002**, *84*, 89–100.
10. Kurath, G.; Leong, J.C. Characterization of infectious hematopoietic necrosis virus mRNA species reveals a nonvirion rhabdovirus protein. *J. Virol.* **1985**, *53*, 462–468.
11. Schutze, H.; Mundt, E.; Mettenleiter, T.C. Complete genomic sequence of viral hemorrhagic septicemia virus, a fish rhabdovirus. *Virus Genes* **1999**, *19*, 59–65.
12. Hilleman, M.R. Strategies and mechanisms for host and pathogen survival in acute and persistent viral infections. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 14560–14566.
13. LaPatra, S.E.; Lauda, K.A.; Jones, G.R.; Walker, S.C.; Shewmaker, B.S.; Morton, A.W. Characterization of IHNV isolates associated with neurotropism. *Vet. Res.* **1995**, *26*, 433–437.
14. Ghittino, P. Viral hemorrhagic septicemia (VHS) in rainbow trout in Italy. *Ann. N. Y. Acad. Sci.* **1965**, *126*, 468–478.
15. Vestergård Jørgensen, P.E. Egtved virus: Temperature-dependent immune response of trout to infection with low-virulence virus. *J. Fish Dis.* **1982**, *5*, 47–55.
16. St-Hilaire, S.; Ribble, C.; Traxler, G.; Davies, T.; Kent, M.L. Evidence for a carrier state of infectious hematopoietic necrosis virus in chinook salmon *Oncorhynchus tshawytscha*. *Dis. Aquat. Organ.* **2001**, *46*, 173–179.
17. Neukirch, M. Demonstration of persistent viral haemorrhagic septicaemia (VHS) virus in rainbow trout after experimental waterborne infecton. *J. Vet. Med.* **1986**, *33*, 471–476.
18. Hershberger, P.K.; Gregg, J.L.; Grady, C.A.; Taylor, L.; Winton, J.R. Chronic and persistent viral hemorrhagic septicemia virus infections in Pacific herring. *Dis. Aquat. Organ.* **2010**, *93*, 43–49.
19. Amend, D.F. Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. *J. Wildl. Dis.* **1975**, *11*, 471–478.
20. Engelking, H.M.; Harry, J.B.; Leong, J.C. Comparison of representative strains of infectious hematopoietic necrosis virus by serological neutralization and cross-protection assays. *Appl. Environ. Microbiol.* **1991**, *57*, 1372–1378.
21. Lorenzen, N.; Lorenzen, E.; Einer-Jensen, K.; Heppell, J.; Wu, T.; Davis, H.L. Genetic vaccination of rainbow trout against viral haemorrhagic septicaemia virus: Small amounts of plasmid DNA protect against a heterologous serotype. *Virus Res.* **1999**, *63*, 19–25.
22. Janeway, C.A.; Travers, P.; Walport, M.; Capra, D.J. *Immunobiology*, 4th ed.; Garland Publishing: New York, NY, USA, 1999; p. 635.

23. Lorenzen, N.; Olesen, N. Immunization with viral antigens: Viral haemorrhagic septicaemia. *Dev. Biol. Stand.* **1997**, *90*, 201–209.
24. Winton, J.R. Immunization with viral antigens: Infectious haematopoietic necrosis. *Dev. Biol. Stand.* **1997**, *90*, 211–220.
25. Lorenzen, N.; LaPatra, S.E. DNA vaccines for aquacultured fish. *Revue Scientifique Et Technique-Office International Des Epizooties* **2005**, *24*, 201–213.
26. Lorenzen, N. Recombinant vaccines: Experimental and applied aspects. *Fish Shellfish Immunol.* **1999**, *9*, 361–365.
27. Kurath, G. Overview of recent DNA vaccine development for fish. *Dev. Biol. (Basel)* **2005**, *121*, 201–213.
28. Salonius, K.; Simard, N.; Harland, R.; Ulmer, J.B. The road to licensure of a DNA vaccine. *Curr. Opin. Investig. Drugs* **2007**, *8*, 635–641.
29. Tonheim, T.C.; Bogwald, J.; Dalmo, R.A. What happens to the DNA vaccine in fish? A review of current knowledge. *Fish Shellfish Immunol.* **2008**, *25*, 1–18.
30. Alonso, M.; Chiou, P.P.; Leong, J.A. Development of a suicidal DNA vaccine for infectious hematopoietic necrosis virus (IHNV). *Fish Shellfish Immunol.* **2011**, *30*, 815–823.
31. Alonso, M.; Johnson, M.; Simon, B.; Leong, J.A. A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. *Vaccine* **2003**, *21*, 1591–1600.
32. Anderson, E.D.; Mourich, D.V.; Fahrenkrug, S.C.; La Patra, S.E.; Shepard, J.; Leong, J.C. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Mol. Mar. Biol. Biotechnol.* **1996**, *5*, 114–122.
33. Lorenzen, E.; Lorenzen, E.; Einer-Jensen, K.; Heppell, J.; Wu, T.; Davis, H.L. Protective immunity to VHS in rainbow trout (*Oncorhynchus mykiss* Walbaum) following DNA vaccination. *Fish Shellfish Immunol.* **1998**, *8*, 261–270.
34. Traxler, G.S.; Anderson, E.; LaPatra, S.E.; Richard, J.; Shewmaker, B.; Kurath, G. Naked DNA vaccination of Atlantic salmon *Salmo salar* against IHNV. *Dis. Aquat. Organ.* **1999**, *38*, 183–190.
35. Emmenegger, E.J.; Kurath, G. DNA vaccine protects ornamental koi (*Cyprinus carpio koi*) against North American spring viremia of carp virus. *Vaccine* **2008**, *26*, 6415–6421.
36. Takano, T.; Iwahori, A.; Hirono, I.; Aoki, T. Development of a DNA vaccine against hirame rhabdovirus and analysis of the expression of immune-related genes after DNA vaccination. *Fish Shellfish Immunol.* **2004**, *17*, 367–374.
37. Lorenzen, N.; Lorenzen, E.; Einer-Jensen, K.; Lapatra, S.E. DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. *Fish Shellfish Immunol.* **2002**, *12*, 439–453.
38. Kurath, G.; Purcell, M.K.; Garver, K.A. Fish rhabdovirus models for understanding host response to DNA vaccines. *CAB Reviews* **2007**, *2*, 1–12.
39. Lorenzen, N.; Lorenzen, E.; Einer-Jensen, K.; La Patra, S.E. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. *Dev. Comp. Immunol.* **2002**, *26*, 173–179.

40. LaPatra, S.E.; Corbeil, S.; Jones, G.R.; Shewmaker, W.D.; Lorenzen, N.; Anderson, E.D.; Kurath, G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. *Vaccine* **2001**, *19*, 4011–4019.
41. Sommerset, I.; Lorenzen, E.; Lorenzen, N.; Bleie, H.; Nerland, A.H. A DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus challenge in turbot. *Vaccine* **2003**, *21*, 4661–4667.
42. Encinas, P.; Gomez-Sebastian, S.; Nunez, M.; Gomez-Casado, E.; Escribano, J.; Estepa, A.; Coll, J. Antibody recognition of the glycoprotein g of viral haemorrhagic septicemia virus (VHSV) purified in large amounts from insect larvae. *BMC Res. Notes* **2011**, *4*, 210.
43. Adelmann, M.; Köllner, B.; Bergmann, S.M.; Fischer, U.; Lange, B.; Weitschies, W.; Enzmann, P.-J.; Fichtner, D. Development of an oral vaccine for immunisation of rainbow trout (*Oncorhynchus mykiss*) against viral haemorrhagic septicaemia. *Vaccine* **2008**, *26*, 837–844.
44. Plant, K.P.; LaPatra, S.E. Advances in fish vaccine delivery. *Dev. Comp. Immunol.* **2011**, *35*, 1256–1262.
45. Kawai, T.; Akira, S. Toll-like receptor and Rig-1 like receptor signaling. *Ann. N. Y. Acad. Sci.* **2008**, *1143*, 1–20.
46. Mogensen, T.H.; Paludan, S.R. Reading the viral signature by Toll-like receptors and other pattern recognition receptors. *J. Mol. Med.* **2005**, *83*, 180–192.
47. Lund, J.M.; Alexopoulou, L.; Sato, A.; Karow, M.; Adams, N.C.; Gale, N.W.; Iwasaki, A.; Flavell, R.A. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 5598–5603.
48. Georgel, P.; Jiang, Z.F.; Kunz, S.; Janssen, E.; Mols, J.; Hoebe, K.; Bahram, S.; Oldstone, M.B.; Beutler, B. Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway. *Virology* **2007**, *362*, 304–313.
49. Edelmann, K.H.; Richardson-Burns, S.; Alexopoulou, L.; Tyler, K.L.; Flavell, R.A.; Oldstone, M.B. Does Toll-like receptor 3 play a biological role in virus infections? *Virology* **2004**, *322*, 231–238.
50. Faul, E.J.; Wanjalla, C.N.; Suthar, M.S.; Gale, M.; Wirblich, C.; Schnell, M.J. Rabies virus infection induces type I interferon production in an IPS-1 dependent manner while dendritic cell activation relies on IFNAR signaling. *PLoS Pathog.* **2010**, *6*, e1001016.
51. Kato, H.; Takeuchi, O.; Sato, S.; Yoneyama, M.; Yamamoto, M.; Matsui, K.; Uematsu, S.; Jung, A.; Kawai, T.; Ishii, K.J.; *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **2006**, *441*, 101–105.
52. Samuel, C. Antiviral actions of interferons. *Clin. Microbiol. Rev.* **2001**, *14*, 778–809.
53. Ank, N.; West, H.; Paludan, S.R. IFN-lambda: Novel antiviral cytokines. *J. Interferon Cytokine Res.* **2006**, *26*, 373–379.
54. Rieder, M.; Conzelmann, K.K. Rhabdovirus evasion of the interferon system. *J. Interferon Cytokine Res.* **2009**, *29*, 499–509.
55. Komatsu, T.; Bi, Z.; Reiss, C.S. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. *J. Neuroimmunol.* **1996**, *68*, 101–108.
56. D'Agostino, P.M.; Yang, J.; Reiss, C.S. Distinct mechanisms of inhibition of VSV replication in neurons mediated by type I and type II IFN. *Virus Rev. Res.* **2009**, *14*, 20–29.

57. Sen, G.C. Viruses and interferons. *Annu. Rev. Microbiol.* **2001**, *55*, 255–281.
58. Kalvakolanu, D.V. Alternate interferon signaling pathways. *Pharmacol. Therapeut.* **2003**, *100*, 1–29.
59. van Boxel-Dezaire, A.H.; Rani, M.R.; Stark, G.R. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* **2006**, *25*, 361–372.
60. MacMicking, J.D. IFN-inducible GTPases and immunity to intracellular pathogens. *Trends Immunol.* **2004**, *25*, 601–609.
61. Balachandran, S.; Roberts, P.C.; Brown, L.E.; Truong, H.; Pattnaik, A.K.; Archer, D.R.; Barber, G.N. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* **2000**, *13*, 129–141.
62. Thomsen, A.R.; Nansen, A.; Andersen, C.; Johansen, J.; Marker, O.; Christensen, J.P. Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus. *Int. Immunol.* **1997**, *9*, 1757–1766.
63. Freer, G.; Burkhart, C.; Ciernik, I.; Bachmann, M.F.; Hengartner, H.; Zinkernagel, R.M. Vesicular stomatitis virus Indiana glycoprotein as a T-cell-dependent and -independent antigen. *J. Virol.* **1994**, *68*, 3650–3655.
64. Zou, J.; Bird, S.; Secombes, C. Antiviral sensing in teleost fish. *Curr. Pharmaceut. Des.* **2010**, *16*, 4185–4193.
65. Palti, Y. Toll-like receptors in bony fish: From genomics to function. *Dev. Comp. Immunol.* **2011**, *35*, 1263–1272.
66. Rebl, A.; Goldammer, T.; Seyfert, H.-M. Toll-like receptor signaling in bony fish. *Vet. Immunol. Immunopathol.* **2010**, *134*, 139–150.
67. Huang, T.; Su, J.; Heng, J.; Dong, J.; Zhang, R.; Zhu, H. Identification and expression profiling analysis of grass carp *Ctenopharyngodon idella* LGP2 cDNA. *Fish Shellfish Immunol.* **2010**, *29*, 349–355.
68. Su, J.; Huang, T.; Dong, J.; Heng, J.; Zhang, R.; Peng, L. Molecular cloning and immune responsive expression of MDA5 gene, a pivotal member of the RLR gene family from grass carp *Ctenopharyngodon idella*. *Fish Shellfish Immunol.* **2010**, *28*, 712–718.
69. Biacchesi, S.; LeBerre, M.; Lamoureux, A.; Louise, Y.; Lauret, E.; Boudinot, P.; Bremont, M. Mitochondrial antiviral signaling protein plays a major role in induction of the fish innate immune response against RNA and DNA viruses. *J. Virol.* **2009**, *83*, 7815–7827.
70. Skjaeveland, I.; Iliev, D.B.; Strandskog, G.; Jorgensen, J.B. Identification and characterization of TLR8 and MyD88 homologs in Atlantic salmon (*Salmo salar*). *Dev. Comp. Immunol.* **2009**, *33*, 1011–1017.
71. Palti, Y.; Gahr, S.A.; Purcell, M.K.; Hadidi, S.; Rexroad, C.E., 3rd; Wiens, G.D. Identification, characterization and genetic mapping of TLR7, TLR8a1 and TLR8a2 genes in rainbow trout (*Oncorhynchus mykiss*). *Dev. Comp. Immunol.* **2009**, *34*, 219–233.
72. Rodriguez, M.F.; Wiens, G.; Purcell, M.K.; Palti, Y. Characterization of Toll-like receptor 3 gene in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics* **2005**, *57*, 510–519.
73. Matsuo, A.; Oshiumi, H.; Tsujita, T.; Mitani, H.; Kasai, H.; Yoshimizu, M.; Matsumoto, M.; Seya, T. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *J. Immunol.* **2008**, *181*, 3474–3485.

74. Sullivan, C.; Charette, J.; Catchen, J.; Lage, C.R.; Giasson, G.; Postlethwait, J.H.; Millard, P.J.; Kim, C.H. The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions. *J. Immunol.* **2009**, *183*, 5896–5908.
75. Verjan, N.; Ooi, E.L.; Nochi, T.; Kondo, H.; Hirono, I.; Aoki, T.; Kiyono, H.; Yuki, Y. A soluble nonglycosylated recombinant infectous hematopoietic necrosis virus (IHNV) G-protein induces IFNs in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **2008**, *25*, 170–180.
76. Acosta, F.; Collet, B.; Lorenzen, N.; Ellis, A.E. Expression of the glycoprotein of viral haemorrhagic septicaemia virus (VHSV) on the surface of the fish cell line RTG-P1 induces type 1 interferon expression in neighbouring cells. *Fish Shellfish Immunol.* **2006**, *21*, 272–278.
77. Chico, V.; Martinez-Lopez, A.; Ortega-Villaizan, M.; Falco, A.; Perez, L.; Coll, J.M.; Estepa, A. Pepscan mapping of viral hemorrhagic septicemia virus glycoprotein G major lineal determinants implicated in triggering host cell antiviral responses mediated by type I interferon. *J. Virol.* **2010**, *84*, 7140–7150.
78. Chang, M.; Collet, B.; Nie, P.; Lester, K.; Campbell, S.; Secombes, C.J.; Zou, J. Expression and functional characterization of the RIG-I-like receptors MDA5 and LGP2 in rainbow trout (*Oncorhynchus mykiss*). *J. Virol.* **2011**, *85*, 8403–8412.
79. Altman, S.M.; Mellon, M.T.; Distel, D.L.; Kim, C.H. Molecular and functional analysis of an interferon gene from the zebrafish, *Danio rerio*. *J. Virol.* **2003**, *77*, 1992–2002.
80. Long, S.; Wilson, M.; Bengten, E.; Bryan, L.; Clem, L.W.; Miller, N.W.; Chinchar, V.G. Identification of a cDNA encoding channel catfish interferon. *Dev. Comp. Immunol.* **2004**, *28*, 97–111.
81. Long, S.; Milev-Milovanovic, I.; Wilson, M.; Bengten, E.; Clem, L.W.; Miller, N.W.; Chinchar, V.G. Identification and expression analysis of cDNAs encoding channel catfish type I interferons. *Fish Shellfish Immunol.* **2006**, *21*, 42–59.
82. Robertsen, B.; Bergan, V.; Rokenes, T.; Larsen, R.; Albuquerque, A. Atlantic salmon interferon genes: Cloning, sequence analysis, expression, and biological activity. *J. Interferon Cytokine Res.* **2003**, *10*, 601–612.
83. Sun, B.; Robertsen, B.; Wang, Z.; Bin, L. Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties. *Dev. Comp. Immunol.* **2008**, *33*, 547–558.
84. Zou, J.; Tafalla, C.; Truckle, J.; Secombes, C. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. *J. Immunol.* **2007**, *179*, 3859–3871.
85. Purcell, M.K.; Laing, K.J.; Woodson, J.; Thorgaard, G.; Hansen, J.D. Characterization of the interferon genes in homozygous rainbow trout reveals two novel genes, alternate splicing and differential regulation of duplicated genes. *Fish Shellfish Immunol.* **2009**, *26*, 293–304.
86. Milev-Milovanovic, I.; Long, S.; Wilson, M.; Bengten, E.; Miller, N.W.; Chinchar, V.G. Identification and expression analysis of interferon gamma genes in channel catfish. *Immunogenetics* **2006**, *58*, 70–80.
87. Zou, J.; Carrington, A.C.; Collet, B.; Dijkstra, J.M.; Yoshiura, Y.; Bols, N.; Secombes, C.J. Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: The first Th1-type cytokine characterized functionally in fish. *J. Immunol.* **2005**, *175*, 2484–2494.

88. Igawa, D.; Sakai, M.; Savan, R. An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals. *Mol. Immunol.* **2006**, *43*, 999–1009.
89. Stein, C.; Caccamo, M.; Laird, G.; Leptin, M. Conservation and divergence of gene families encoding components of innate immune response systems in zebrafish. *Genome Biol.* **2007**, *8*, R251.
90. Levraud, J.P.; Boudinot, P.; Colin, I.; Benmansour, A.; Peyrieras, N.; Herbomel, P.; Lutfalla, G. Identification of the zebrafish IFN receptor: Implications for the origin of the vertebrate IFN system. *J. Immunol.* **2007**, *178*, 4385–4394.
91. Aggad, D.; Mazel, M.; Boudinot, P.; Mogensen, K.E.; Hamming, O.J.; Hartmann, R.; Kotenko, S.; Herbomel, P.; Lutfalla, G.; Levraud, J.P. The two groups of zebrafish virus-induced interferons signal via distinct receptors with specific and shared chains. *J. Immunol.* **2009**, *183*, 3924–3931.
92. Zou, J.; Secombes, C.J. Teleost fish interferons and their role in immunity. *Dev. Comp. Immunol.* **2011**, *2011*, 1376–1387.
93. de Kinkelin, P.; Dorson, M. Interferon production in rainbow trout (*Salmo gairdneri*) experimentally infected with Egtved Virus. *J. Gen. Virol.* **1973**, *19*, 125–127.
94. de Kinkelin, P.; Dorson, M.; Hattenberger-Baudouy, A.M. Interferon synthesis in trout and carp after viral infection. *Dev. Comp. Immunol.* **1982**, *2*, 167–174.
95. Eaton, W.D. Anti-viral activity in four species of salmonids following exposure to poly inosinic: Cytidylic acid. *Dis. Aquat. Organ.* **1990**, *9*, 193–198.
96. Wang, L.; Zhang, H.X.; Zhang, J.H.; Chen, W.H.; Ruan, X.F.; Xia, C. *In vitro* effects of recombinant zebrafish IFN on spring viremia of carp virus and infectious hematopoietic necrosis virus. *J. Interferon Cytokine Res.* **2006**, *26*, 256–259.
97. Ooi, E.L.; Verjan, N.; Haraguchi, I.; Oshima, T.; Kondo, H.; Hirono, I.; Aoki, T.; Kiyono, H.; Yuki, Y. Innate immunomodulation with recombinant interferon-alpha enhances resistance of rainbow trout (*Oncorhynchus mykiss*) to infectious hematopoietic necrosis virus. *Dev. Comp. Immunol.* **2008**, *32*, 1211–1220.
98. de Kinkelin, P.; Le Berre, M. Hematopoietic infectious necrosis of Salmonidae: Production of circulating interferon after experimental infection of the rainbow trout (*Salmo gairdneri*). *C. R. Acad. Sci. Hebd. Seances Acad. Sci. D.* **1974**, *279*, 445–448.
99. Trobridge, G.D.; Chiou, P.P.; Leong, J.C. Cloning of the rainbow trout (*Oncorhynchus mykiss*) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells. *J. Virol.* **1997**, *71*, 5304–5311.
100. O'Farrell, C.; Vaghefi, N.; Cantonnet, M.; Buteau, B.; Boudinot, P.; Benmansour, A. Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of interferon-responsive genes in the early response to a rhabdovirus infection. *J. Virol.* **2002**, *76*, 8040–8049.
101. Purcell, M.K.; Kurath, G.; Garver, K.A.; Herwig, R.P.; Winton, J.R. Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish Shellfish Immunol.* **2004**, *17*, 447–462.
102. Collet, B.; Boudinot, P.; Benmansour, A.; Secombes, C.J. An Mx1 promoter-reporter system to study interferon pathways in rainbow trout. *Dev. Comp. Immunol.* **2004**, *28*, 793–801.

103. Lopez-Munoz, A.; Roca, F.J.; Meseguer, J.; Mulero, V. New insights into the evolution of IFNs: Zebrafish group II IFNs induce a rapid and transient expression of IFN-dependent genes and display powerful antiviral activities. *J. Immunol.* **2009**, *182*, 3440–3449.
104. Chaves-Pozo, E.; Zou, J.; Secombes, C.J.; Cuesta, A.; Tafalla, C. The rainbow trout (*Oncorhynchus mykiss*) interferon response in the ovary. *Mol. Immunol.* **2010**, *47*, 1757–1764.
105. Martin, S.A.; Taggart, J.B.; Seear, P.; Bron, J.E.; Talbot, R.; Teale, A.J.; Sweeney, G.E.; Hoyheim, B.; Houlihan, D.F.; Tocher, D.R.; *et al.* Interferon type I and type II responses in an Atlantic salmon (*Salmo salar*) SHK-1 cell line by the salmon TRAITS/SGP microarray. *Physiol. Genom.* **2007**, *32*, 33–44.
106. Sun, B.; Skjaeveland, I.; Svingerud, T.; Zou, J.; Jorgensen, J.; Robertsen, B. Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon. *J. Virol.* **2011**, *85*, 9188–9198.
107. Verrier, E.R.; Langevin, C.; Benmansour, A.; Boudinot, P. Early antivirial response and virus-induced genes in fish. *Dev. Comp. Immunol.* **2011**, *35*, 1204-1214.
108. Byon, J.Y.; Ohira, T.; Hirono, I.; Aoki, T. Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. *Fish Shellfish Immunol.* **2005**, *18*, 135–147.
109. Byon, J.Y.; Ohira, T.; Hirono, I.; Aoki, T. Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. *Vaccine* **2006**, *24*, 921–930.
110. Purcell, M.K.; Nichols, K.M.; Winton, J.R.; Kurath, G.; Thorgaard, G.H.; Wheeler, P.; Hansen, J.D.; Herwig, R.P.; Park, L.K. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against *Infectious hematopoietic necrosis virus*. *Mol. Immunol.* **2006**, *43*, 2089–2106.
111. Peñaranda, M.M.D.; Purcell, M.K.; Kurath, G. Differential virulence mechanisms of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*) include host entry and virus replication kinetics. *J. Gen. Virol.* **2009**, *90*, 2172–2182.
112. Purcell, M.K.; Garver, K.A.; Conway, C.M.; Elliott, D.G.; Kurath, G. Infectious hematopoietic necrosis virus genogroup-specfic virulence mechanisms in sockeye salmon (*Oncorhynchus nerka*) from Redfish Lake Idaho. *J. Fish Dis.* **2009**, *32*, 619–631.
113. Bernard, J.; Bearzotti-le Berre, M.; De Kinkelin, P. Viral haemorrhagic septicaemia in rainbow trout: Attempt to relate interferon production, antibody synthesis and structure of the virus with the mechanism of virulence. *Annalles Institute Pasteur* **1985**, *136*, 13–26.
114. Park, J.W.; Moon, C.H.; Wargo, A.W.; Purcell, M.K.; Kurath, G. Differential growth of U and M type infectious hematopoietic necrosis virus in a rainbow trout-derived cell line, RTG-2. *J. Fish Dis.* **2010**, *33*, 583–591.
115. Wargo, A.R.; Garver, K.A.; Kurath, G. Virulence correlates with fitness *in vivo* for two M group genotypes of Infectious hematopoietic necrosis virus (IHNV). *Virology* **2010**, *404*, 51–58.
116. Wargo, A.R.; Kurath, G. *In vivo* fitness associated with high virulence in a vertebrate virus is a complex trait regulated by host entry, replication, and shedding. *J. Virol.* **2011**, *85*, 3959–3967.

117. Purcell, M.K.; Lapatra, S.E.; Woodson, J.C.; Kurath, G.; Winton, J.R. Early viral replication and induced or constitutive immunity in rainbow trout families with differential resistance to Infectious hematopoietic necrosis virus (IHNV). *Fish Shellfish Immunol.* **2009**, *28*, 98–105.
118. Dorson, M.; Torhy, C. Viral haemorrhagic septicaemia virus replication in external tissue excised from rainbow trout, *Oncorhynchus mykiss* (Walbaum), and hybrids of different susceptibilities. *J. Fish Dis.* **1993**, *16*, 403–408.
119. Quillet, E.; Dorson, M.; Aubard, G.; Torhy, C. *In vitro* viral haemorrhagic septicemia virus replication in excised fins of rainbow trout: Correlation with resistance to waterborne challenge and genetic variation. *Dis. Aquat. Organ.* **2001**, *45*, 171–182.
120. Quillet, E.; Dorson, M.; Aubard, G.; Torhy, C. *In vitro* assay to select rainbow trout with variable resistance/susceptibility to viral haemorrhagic septicaemia virus. *Dis. Aquat. Organ.* **2007**, *76*, 7–16.
121. Harmache, A.; LeBerre, M.; Droineau, S.; Giovannini, M.; Bremont, M. Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for *Novirhabdovirus*. *J. Virol.* **2006**, *80*, 3655–3659.
122. Lorenzen, N.; LaPatra, S.E. Immunity to rhabdoviruses in rainbow trout: The antibody response. *Fish Shellfish Immunol.* **1999**, *9*, 345–360.
123. LaPatra, S.E.; Turner, T.; Lauda, K.A.; Jones, G.R.; Walker, S. Characterization of the humoral response of rainbow trout to infectious hematopoietic necrosis virus. *J. Aquat. Anim. Health* **1993**, *5*, 165–171.
124. LaPatra, S.E.; Lauda, K.A.; Jones, G.R.; Walker, S.; Shewmaker, W.D. Development of passive immunotherapy for control of infectious haematopoietic necrosis. *Dis. Aquat. Organ.* **1994**, *20*, 1–6.
125. Hershberger, P.K.; Gregg, J.L.; Grady, C.A.; LaPatra, S.E.; Winton, J.R. Passive immunization of Pacific herring *Clupea pallasii* against viral hemorrhagic septicemia. *J. Aquat. Anim. Health* **2011**, *23*, 140–147.
126. Lorenzen, N.; Olesen, N.J.; Koch, C. Immunity to VHS virus in rainbow trout. *Aquaculture* **1999**, *172*, 41–61.
127. Kurath, G.; Garver, K.A.; LaPatra, S.E.; Purcell, M.K. Resistance and protective immunity in Redfish Lake sockeye salmon exposed to M type infectious hematopoietic necrosis virus (IHNV). *J. Aquat. Anim. Health* **2010**, *22*, 129–139.
128. McLauchlan, P.E.; Collet, B.; Ingerslev, E.; Secombes, C.J.; Lorenzen, N.; Ellis, A.E. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: Size, dose, route of injections and duration of protection-early protection correlates with Mx expression. *Fish Shellfish Immunol.* **2003**, *15*, 39–50.
129. Corbeil, S.; LaPatra, S.E.; Anderson, E.D.; Kurath, G. Nanogram quantities of a DNA vaccine protect rainbow trout fry against heterologous virus strains of infectious hematopoietic necrosis virus. *Vaccine* **2000**, *18*, 2817–2824.
130. LaPatra, S.E.; Corbeil, S.; Jones, G.R.; Shewmaker, W.D.; Kurath, G. The dose-dependent effect on protection and humoral response to a DNA vaccine against IHN virus in subyearling rainbow trout. *J. Aquat. Anim. Health* **2000**, *12*, 181–188.

131. Garver, K.A.; LaPatra, S.E.; Kurath, G. Efficacy of an infectious hematopoietic necrosis (IHN) virus DNA vaccine in Chinook *Oncorhynchus tshawytscha* and sockeye *O. nerka* salmon. *Dis. Aquat. Organ.* **2005**, *64*, 13–22.
132. Boudinot, P.; Blanco, M.; de Kinkelin, P.; Benmansour, A. Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoeitic necrosis virus induces double-specific immunity and nonspecific responses in rainbow trout. *Virology* **1998**, *249*, 297–306.
133. Kurath, G.; Garver, K.A.; Corbeil, S.; Elliott, D.G.; Anderson, E.D.; LaPatra, S.E. Protective immunity and lack of histopathological damage two years after DNA vaccination against infectious hematopoietic necrosis virus in trout. *Vaccine* **2006**, *16*, 345–354.
134. Li, J.; Barreda, D.R.; Zhang, Y.A.; Boshra, H.; Gelman, A.E.; Lapatra, S.; Tort, L.; Sunyer, J.O. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat. Immunol.* **2006**, *7*, 1116–1124.
135. Hansen, J.D.; Landis, E.D.; Phillips, R.B. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 6919–6924.
136. Danilova, N.; Bussmann, J.; Jekosch, K.; Steiner, L.A. The immunoglobulin heavy-chain locus in zebrafish: Identification and expression of a previously unknown isotype, immunoglobulin Z. *Nat. Immunol.* **2005**, *6*, 295–302.
137. Hordvik, I.; Berven, F.S.; Solem, S.T.; Hatten, F.; Endresen, C. Analysis of two IgM isotypes in Atlantic salmon and brown trout. *Mol. Immunol.* **2002**, *39*, 313–321.
138. Zhang, Y.A.; Salinas, I.; Li, J.; Parra, D.; Bjork, S.; Xu, Z.; LaPatra, S.E.; Bartholomew, J.; Sunyer, J.O. IgT, a primitive immunoglobulin class specialized in mucosal immunity. *Nat. Immunol.* **2010**, *11*, 827–835.
139. DeLuca, D.; Wilson, M.; Warr, G.W. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. *Eur. J. Immunol.* **1983**, *13*, 546–551.
140. Nakao, M.; Tsujikura, M.; Ichiki, S.; Vo, T.K.; Somamoto, T. The complement system in teleost fish: Progress of post-homolog-hunting researches. *Dev. Comp. Immunol.* **2011**, *35*, 1296–1308.
141. Laing, K.J.; Hansen, J.D. Fish T cells: Recent advances through genomics. *Dev. Comp. Immunol.* **2011**, *35*, 1282–1295.
142. Miller, K.; Traxler, G.; Kaukinen, K.; Li, S.; Richard, J.; Ginther, N. Salmonid host response to infectious hematopoietic necrosis (IHN) virus: Cellular receptors, viral control, and novel pathways of defense. *Aquaculture* **2007**, *272*, S217–S237
143. Boudinot, P.; Bernard, D.; Boubekeur, S.; Thoulouze, M.-I.; Bremont, M.; Benmansour, A. The glycoprotein of a fish rhabdovirus profiles the virus-specific T-cell repertoire in rainbow trout. *J. Gen. Virol.* **2004**, *85*, 3099–3108.
144. Boudinot, P.; Boubekeur, S.; Benmansour, A. Rhabdovirus infection induces public and private T cell responses in teleost fish. *J. Immunol.* **2001**, *167*, 6202–6209.
145. Takizawa, F.; Dijkstra, J.M.; Kotterba, P.; Korytar, T.; Kock, H.; Kollner, B.; Jaureguiberry, B.; Nakanishi, T.; Fischer, U. The expression of CD8 alpha discriminates distinct T cell subsets in teleost fish. *Dev. Comp. Immunol.* **2011**, *35*, 752–763.

146. Nakanishi, T.; Toda, H.; Shibasaki, Y.; Somamoto, T. Cytotoxic T cells in teleost fish. *Dev. Comp. Immunol.* **2011**, *35*, 1317–1323.
147. Fischer, U.; Utke, K.; Somamoto, T.; Kollner, B.; Ototake, M.; Nakanishi, T. Cytotoxic activities of fish leucocytes. *Fish Shellfish Immunol.* **2006**, *20*, 209–226.
148. Evans, D.E.; Jaso-Friedman, L. Nonspecific cytotoxic cells as effectors of immunity in fish. *Annu. Rev. Fish Dis.* **1992**, *2*, 109–121.
149. Shen, L.L.; Stuge, T.B.; Evenhuis, J.P.; Bengten, E.; Wilson, M.; Chinchar, V.G.; Clem, L.W.; Miller, N.W. Channel catfish NK-like cells are armed with IgM via a putative FcmuR. *Dev. Comp. Immunol.* **2003**, *27*, 699–714.
150. Shen, L.; Stuge, T.B.; Bengten, E.; Wilson, M.; Chinchar, V.G.; Naftel, J.P.; Bernanke, J.M.; Clem, L.W.; Miller, N.W. Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.* **2004**, *28*, 139–152.
151. Kurata, O.; Okamoto, N.; Ikeda, Y. Neutrophilic granulocytes in carp, *Cyprinus carpio*, possess a spontaneous cytotoxic activity. *Dev. Comp. Immunol.* **1995**, *19*, 315–325.
152. Yoder, J.A.; Litman, G.W. The phylogenetic origins of natural killer receptors and recognition: Relationships, possibilities, and realities. *Immunogenetics* **2011**, *63*, 123–141.
153. Dijkstra, J.M.; Kollner, B.; Aoyagi, K.; Sawamoto, Y.; Kuroda, A.; Ototake, M.; Nakanishi, T.; Fischer, U. The rainbow trout classical MHC class I molecule Onmy-UBA*501 is expressed in similar cell types as mammalian classical MHC class I molecules. *Fish Shellfish Immunol.* **2003**, *14*, 1–23.
154. Utke, K.; Bergmann, S.; Lorenzen, N.; Kollner, B.; Ototake, M.; Fischer, U. Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. *Fish Shellfish Immunol.* **2007**, *22*, 182–196.
155. Somamoto, T.; Nakanishi, T.; Okamoto, N. Role of specific cell-mediated cytotoxicity in protecting fish from viral infections. *Virology* **2002**, *297*, 120–127.
156. Le Morvan, C.; Troutaud, D.; Deschaux, P. Differential effects of temperature on specific and nonspecific immune defenses in fish. *J. Exp. Biol.* **1998**, *201*, 165–168.
157. Fijan, N. Spring viraemia of carp and other viral diseases and agents of warm-water fish. In *Fish Diseases and Disorders*; Woo, P.T.K., Bruno, D.W., Eds.; CAB International: Wallingford, UK, 1999; Volume 3, pp. 177–244.
158. Wolf, K. Viral hemorrhagic septicemia. In *Fish Viruses and Fish Viral Diseases*; Cornell University Press: Ithaca, NY, USA, 1988; pp. 217–249.
159. Amend, D.F. Prevention and control of viral diseases of salmonids. *J. Fish. Res. Board Can.* **1976**, *33*, 1059–1066.
160. Goodwin, A.E.; Merry, G.E. Mortality and carrier status of bluegills exposed to viral hemorrhagic septicemia virus genotype IVb at different temperatures. *J. Aquat. Anim. Health* **2011**, *23*, 85–91.
161. Amend, D.F. Control of infectious hematopoietic necrosis virus disease by elevating the water temperature. *J. Fish. Res. Board Can.* **1970**, *27*, 265–270.
162. Sano, M.; Ito, M.T.; Nakayasu, C.; Kurita, J. Effect of water temperature shifting on mortality of Japanese flounder *Paralichthys olivaceus* experimentally infected with viral hemorrhagic septicemia virus. *Aquaculture* **2009**, *286*, 254–258.

163. LaPatra, S.E.; Batts, W.N.; Overturf, K.; Jones, G.R.; Shewmaker, W.D.; Winton, J.R. Negligible risk associated with the movement of processed rainbow trout, *Oncorhynchus mykiss* (Walbaum), from an infectious haematopoietic necrosis virus (IHNV) endemic area. *J. Fish Dis.* **2001**, *24*, 399–408.
164. Lorenzen, E.; Einer-Jensen, K.; Rasmussen, J.S.; Kjaer, T.E.; Collet, B.; Secombes, C.J.; Lorenzen, N. The protective mechanisms induced by a fish rhabdovirus DNA vaccine depend on temperature. *Vaccine* **2009**, *27*, 3870–3880.
165. Alcorn, S.W.; Murray, A.L.; Pascho, R.J. Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). *Fish Shellfish Immunol.* **2002**, *12*, 303–334.
166. Utke, K.; Kock, H.; Schuetze, H.; Bergmann, S.M.; Lorenzen, N.; Einer-Jensen, K.; Kollner, B.; Dalmo, R.A.; Vesely, T.; Ototake, M.; *et al.* Cell-mediated immune responses in rainbow trout after DNA immunization against the viral hemorrhagic septicemia virus. *Dev. Comp. Immunol.* **2008**, *32*, 239–252.
167. Sealey, W.M.; Barrows, F.T.; Johansen, K.A.; Overturf, K.; LaPatra, S.E.; Hardy, R.W. Evaluation of the ability of partially autolyzed yeast and Grobiotic-A to improve disease resistance in rainbow trout. *N. Am. J. Aquaculture* **2007**, *69*, 400–406.
168. Beaulaurier, J.; Bickford, N.; Gregg, J.L.; Grady, C.A.; Gannam, A.; Winton, J.R.; Hershberger, P.K. Susceptibility of Pacific herring Clupea pallasii to viral hemorrhagic septicemia (VHS) is influenced by diet. *J. Aquat. Anim. Health* **2012**, in press.
169. Lyles, D.S. Cytopathogenesis and inhibition of host gene expression by RNA viruses. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 709–724.
170. Ahmed, M.; Lyles, D.S. Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III. *J. Virol.* **1998**, *72*, 8413–8419.
171. Rieder, M.; Brzozka, K.; Pfaller, C.K.; Cox, J.H.; Stitz, L.; Conzelmann, K.K. Genetic dissection of interferon-antagonistic functions of rabies virus phosphoprotein: Inhibition of interferon regulatory factor 3 activation is important for pathogenicity. *J. Virol.* **2011**, *85*, 842–852.
172. Blondel, D.; Regad, T.; Poisson, N.; Pavie, B.; Harper, F.; Pandolfi, P.P.; De The, H.; Chelbi-Alix, M.K. Rabies virus P and small P products interact directly with PML and reorganize PML nuclear bodies. *Oncogene* **2002**, *21*, 7957–7970.
173. Petersen, J.M.; Her, L.S.; Varvel, V.; Lund, E.; Dahlberg, J.E. The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes. *Mol. Biol. Cell* **2000**, *20*, 8590–8601.
174. Connor, J.H.; Lyles, D.S. Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation. *J. Biol. Chem.* **2005**, *280*, 13512–13519.
175. Benedict, C.A.; Norris, P.S.; Ware, C.F. To kill or be killed: Viral evasion of apoptosis. *Nat. Immunol.* **2002**, *3*, 1013–1018.
176. Gaddy, D.F.; Lyles, D.S. Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *J. Virol.* **2005**, *79*, 4170–4179.
177. Kopecky, S.A.; Willingham, M.C.; Lyles, D.S. Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. *J. Virol.* **2001**, *75*, 12169–12181.

178. Kopecky, S.A.; Lyles, D.S. Contrasting effects of matrix protein on apoptosis in HeLa and BHK cells infected with vesicular stomatitis virus are due to inhibition of host gene expression. *J. Virol.* **2003**, *77*, 4658–4669.
179. Koyama, A.H. Induction of apoptotic DNA fragmentation by the infection of vesicular stomatitis virus. *Virus Res.* **1995**, *37*, 285–290.
180. Frank, S.A. *Immunology and Evolution of Infectious Disease*; Princeton University Press: Princeton, NJ, USA, 2002; p. 348.
181. Benmansour, A.; Leblois, H.; Coulon, P.; Tuffereau, C.; Gaudin, Y.; Flamand, A.; Lafay, F. Antigenicity of rabies virus glycoprotein. *J. Virol.* **1991**, *65*, 4198–4203.
182. Luo, L.H.; Li, Y.; Snyder, R.M.; Wagner, R.R. Point mutations in glycoprotein gene of vesicular stomatitis virus (New Jersey serotype) selected by resistance to neutralization by epitope-specific monoclonal antibodies. *Virology* **1988**, *163*, 341–348.
183. Bilsel, P.A.; Nichol, S.T. Polymerase errors accumulating during natural evolution of the glycoprotein gene of vesicular stomatitis virus Indiana serotype isolates. *J. Virol.* **1990**, *64*, 4873–4883.
184. Luo, L.Z.; Li, Y.; Snyder, R.M.; Wagner, R.R. Spontaneous mutations leading to antigenic variations in the glycoproteins of vesicular stomatitis virus field isolates. *Virology* **1990**, *174*, 70–78.
185. Sloan, S.E.; Hanlon, C.; Weldon, W.; Niezgoda, M.; Blanton, J.; Self, J.; Rowley, K.J.; Mandell, R.B.; Babcock, G.J.; Thomas, W.D., Jr.; *et al.* Identification and characterization of a human monoclonal antibody that potently neutralizes a broad panel of rabies virus isolates. *Vaccine* **2007**, *25*, 2800–2810.
186. Chiou, P.P.; Kim, C.H.; Ormonde, P.; Leong, J.A. Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. *J. Virol.* **2000**, *74*, 7619–7627.
187. Ammayappan, A.; Vakharia, V.N. Nonvirion protein of novirhabdovirus suppresses apoptosis at the early stage of virus infection. *J. Virol.* **2011**, *85*, 8393–8402.
188. Johnson, M.C.; Simon, B.E.; Kim, C.H.; Leong, J.-A.C. Production of recombinant snakehead rhabdovirus: The NV protein is not required for viral replication. *J. Virol.* **2000**, *74*, 2343–2350.
189. Biacchesi, S.; Thoulouze, M.I.; Bearzotti, M.; Yu, Y.X.; Bremont, M. Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. *J. Virol.* **2000**, *74*, 11247–11253.
190. Fijan, N.; Sulimanovic, D.; Bearzotti, M.; Muzinic, D.; Zwillenberg, L.; Chilmonczyk, S.; Vautherot, J.F.; de Kinkelin, P. Some properties of the *epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio. Ann. Virol.* **1983**, *134E*, 207–220.
191. Winton, J.; Batts, W.; deKinkelin, P.; LeBerre, M.; Bremont, M.; Fijan, N. Current lineages of the epithelioma papulosum cyprini (EPC) cell line are contaminated with fathead minnow, *Pimephales promelas*, cells. *J. Fish Dis.* **2010**, *33*, 701–704.
192. Alonso, M.; Kim, C.H.; Johnson, M.C.; Pressley, M.; Leong, J.-A. The NV gene of snakehead rhabdovirus (SHRV) is not required for pathogenesis, and a heterologous glycoprotein can be incorporated into the SHRV envelope. *J. Virol.* **2004**, *78*, 5875–5882.

193. Thoulouze, M.-I.; Bouguyon, E.; Carpentier, C.; Bremont, M. Essential Role of the NV Protein of Novirhabdovirus for Pathogenicity in Rainbow Trout. *J. Virol.* **2004**, *78*, 4098–4107.
194. Ammayappan, A.; Kurath, G.; Thompson, T.M.; Vakharia, V.N. A reverse genetics system for the Great Lakes strain of viral hemorrhagic septicemia virus: The NV gene is required for pathogenicity. *Mar. Biotechnol.* **2011**, *13*, 672–683.
195. Choi, M.K.; Moon, C.H.; Ko, M.S.; Lee, U.H.; Cho, W.J.; Cha, S.J.; Do, J.W.; Heo, G.J.; Jeong, S.G.; Hahm, Y.S.; *et al.* A Nuclear Localization of the Infectious Haematopoietic Necrosis Virus NV Protein Is Necessary for Optimal Viral Growth. *PLoS One* **2011**, *6*, e22362.
196. Smail, D.A. Viral haemorrhagic septicaemia. In *Fish Diseases and Disorders: Viral, Bacterial, and Fungal Infections*; Woo, P.T.K., Bruno, D.W., Eds.; CAB International: Wallingford, UK, 1999; Volume 3, pp. 123–147.
197. Huang, C.; Chien, M.S.; Landolt, M.; Batts, W.; Winton, J. Mapping the neutralizing epitopes on the glycoprotein of infectious haematopoietic necrosis virus, a fish rhabdovirus. *J. Gen. Virol.* **1996**, *77*, 3033–3040.
198. Nichol, S.T.; Rowe, J.E.; Winton, J.R. Molecular epizootiology and evolution of the glycoprotein and non-virion protein genes of infectious hematopoietic necrosis virus, a fish rhabdovirus. *Virus Res.* **1995**, *38*, 159–173.
199. Kurath, G.; Garver, K.A.; Troyer, R.M.; Emmenegger, E.J.; Einer-Jensen, K.; Anderson, E.D. Phylogeography of infectious haematopoietic necrosis virus in North Amer*ica. J. Gen. Virol.* **2003**, *84*, 803–814.
200. Troyer, R.M.; LaPatra, S.E.; Kurath, G. Genetic analyses reveal unusually high diversity of infectious haematopoietic necrosis virus in rainbow trout aquaculture. *J. Gen. Virol.* **2000**, *81*, 2823–2832.

Kibenge et al. Virology Journal (2016) 13:3
DOI 10.1186/s12985-015-0459-1

Virology Journal

RESEARCH Open Access

Discovery of variant infectious salmon anaemia virus (ISAV) of European genotype in British Columbia, Canada

Molly JT Kibenge[1], Tokinori Iwamoto[1,5], Yingwei Wang[2], Alexandra Morton[3], Richard Routledge[4] and Frederick SB Kibenge[1*]

Abstract

Background: Infectious salmon anaemia (ISA) virus (ISAV) belongs to the genus *Isavirus*, family *Orthomyxoviridae*. ISAV occurs in two basic genotypes, North American and European. The European genotype is more widespread and shows greater genetic variation and greater virulence variation than the North American genotype. To date, all of the ISAV isolates from the clinical disease, ISA, have had deletions in the highly polymorphic region (HPR) on ISAV segment 6 (ISAV-HPRΔ) relative to ISAV-HPR0, named numerically from ISAV-HPR1 to over ISAV-HPR30. ISA outbreaks have only been reported in farmed Atlantic salmon, although ISAV has been detected by RT-PCR in wild fish. It is recognized that asymptomatically ISAV-infected fish exist. There is no universally accepted ISAV RT-qPCR TaqMan® assay. Most diagnostic laboratories use the primer-probe set targeting a 104 bp-fragment on ISAV segment 8. Some laboratories and researchers have found a primer-probe set targeting ISAV segment 7 to be more sensitive. Other researchers have published different ISAV segment 8 primer-probe sets that are highly sensitive.

Methods: In this study, we tested 1,106 fish tissue samples collected from (i) market-bought farmed salmonids and (ii) wild salmon from throughout British Columbia (BC), Canada, for ISAV using real time RT-qPCR targeting segment 8 and/or conventional RT-PCR with segment 8 primers and segment 6 HPR primers, and by virus isolation attempts using Salmon head kidney (SHK-1 and ASK-2) cell line monolayers. The sequences from the conventional PCR products were compared by multiple alignment and phylogenetic analyses.

Results: Seventy-nine samples were "non-negative" with at least one of these tests in one or more replicates. The ISAV segment 6 HPR sequences from the PCR products matched ISAV variants, HPR5 on 29 samples, one sample had both HPR5 and HPR7b and one matched HPR0. All sequences were of European genotype. In addition, alignment of sequences of the conventional PCR product segment 8 showed they had a single nucleotide mutation in the region of the probe sequence and a 9-nucleotide overlap with the reverse primer sequence of the real time RT-qPCR assay. None of the classical ISAV segment 8 sequences in the GenBank have this mutation in the probe-binding site of the assay, suggesting the presence of a novel ISAV variant in BC. A phylogenetic tree of these sequences showed that some ISAV sequences diverted early from the classical European genotype sequences, while others have evolved separately. All virus isolation attempts on the samples were negative, and thus the samples were considered "negative" in terms of the threshold trigger set for Canadian federal regulatory action; i.e., successful virus isolation in cell culture.

(Continued on next page)

* Correspondence: kibenge@upei.ca
[1]Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, P.E.I. C1A 4P3, Canada
Full list of author information is available at the end of the article

(Continued from previous page)

Conclusions: This is the first published report of the detection of ISAV sequences in fish from British Columbia, Canada. The sequences detected, both of ISAV-HPRΔ and ISAV-HPR0 are of European genotype. These sequences are different from the classical ISAV segment 8 sequences, and this difference suggests the presence of a new ISAV variant of European genotype in BC. Our results further suggest that ISAV-HPRΔ strains can be present without clinical disease in farmed fish and without being detected by virus isolation using fish cell lines.

Keywords: Infectious salmon anaemia virus, ISAV, ISAV variant, European genotype

Background

Infectious salmon anaemia virus (ISAV) is an economically important pathogen of marine-farmed Atlantic salmon (*Salmo salar* L.). The disease infectious salmon anaemia (ISA) is arguably the most feared viral disease of the marine farmed salmon industry because it has continued to cause the Atlantic salmon farming industry severe economic losses in an increasing number of countries for the past 30 years. ISAV is the only species of the genus *Isavirus*, and one of the seven genera of the family *Orthomyxoviridae* that includes the influenza viruses [1–3]. A complete sequence of PB1 gene of a putative koi carp orthomyxovirus was obtained from koi carp in California with 43 % amino acid sequence identity with ISAV [4], and there is also an independent reference to an orthomyxovirus from koi carp [5], and to unknown viruses with morphology consistent with members of family *Orthomyxoviridae* isolated from baitfish in Wisconsin, USA [6]. The taxonomic status of these findings is not known. ISAV occurs in two basic genotypes, North American and European [7, 8]. The European genotype is more widespread [9] and shows greater genetic variation [10, 11] and greater virulence variation [12–14] than the North American genotype. ISA outbreaks have only been reported in farmed Atlantic salmon, although ISAV has been detected by RT-PCR in wild fish (Table 1). It is recognized that asymptomatically ISAV-infected fish exist [15, 16]. Since 2012, only Norway, Canada and Chile have reported ISA outbreaks. The ISA outbreaks reported in Canada have occurred in the Atlantic Ocean in New Brunswick, Nova Scotia, and Newfoundland and Labrador [17].

ISAV has a segmented genome with eight single-stranded RNA segments of negative polarity [1]. The *Orthomyxoviridae* family is known to exhibit high mutation rates, and ISAV occurs in at least 30 recognized HPR variants [9, 18]. When viruses mutate, 'drift variants' arise

Table 1 Timeline (chronological history) of the detection of ISAV in wild fish related to first-time outbreaks of ISA in farmed Atlantic salmon

Year of sample &Test used	Country (location)	Wild fish species with ISAV (reference)	First-time outbreaks of ISA in farmed Atlantic salmon in country (reference)
1998-1999, Virus Isolation & RT-PCR	UK (Scotland)	Sea trout, Brown trout, Atlantic salmon [61]	Scotland, UK in 1998 [47]
2000, RT-PCR	Canada (New Brunswick)	Salmonids [62]	New Brunswick, Canada in 1996 [63]
2000, RT-PCR	UK (Scotland)	Atlantic salmon [49]	
2000, RT-PCR	UK (Scotland)	Sea trout, Brown trout, Atlantic salmon [64]	
2001, RT-PCR	West Greenland fishery	Atlantic salmon [65]	
2001, RT-PCR	USA (Maine)	Atlantic salmon (P. Barbash, cited by [66])	Maine, USA in 2001 [67]
2000-2002, Virus Isolation & RT-PCR	USA (Maine)	Pollock*, Atlantic cod** [66]	
1998; 2001–2003, RT-PCR	Norway (western Norway)	Salmonids (wild trout, Atlantic salmon) [31]	Norway in 1984 [68]
1995-2002, Antibody ELISA	USA (Maine & Massachusetts)	Atlantic salmon [69]	
2010, RT-PCR	Denmark	Atlantic salmon§ [70]	
2010, RT-PCR	Chile (an estuary in southern Chile)	free-living *Salmo salar* (escapees) [15]	Chile in 2007 [71]
	Faroe Islands, Denmark		Faroe Islands, Denmark in 2000 [72]

*Pollock taken from inside a marine cage with ISA-disease salmon was weak RT-PCR positive;
**Atlantic cod taken from a well boat holding salmon from a marine cage with clinically diseased fish was CPE positive on SHK cell culture.
§Danish salmon produced for restocking purposes.

that can escape detection by real-time RT-qPCR tests due to mismatches in the primer-probe binding sites [19]. When a mutation occurs in the precise region that a given primer or probe was designed to anneal, test reliability can be significantly decreased [20] producing inconsistent positive and false-negative readings between replicates [21]. There is no scientific standard for interpretation of high, or inconsistent threshold cycle (C_t) values, and so these kinds of results are interchangeably reported as "negative," "suspicious" or "positive" [22, 23]. For the purposes of this work, we simply designated our results as negative or non-negative.

In Canada, a federally reportable fish disease such as ISA must be confirmed at the Fisheries and Oceans (DFO) Canada National Reference Laboratory [24] through successful virus isolation in cell culture [25]. However, ISAV-HPRΔ strains of low virulence and the non-pathogenic ISAV-HPR0 strains grow poorly or not at all in currently available fish cell lines [15, 26–29]. Gagné and Ritchie [30] report an increasing number of ISAV positive results by RT-PCR in Canada that cannot be confirmed by other diagnostic tests. It is also recognized in Norway that ISAV may be present even when attempts at virus isolation are negative as ISAV has never been isolated from a wild salmon despite positive RT-PCR results (Table 1) [31].

While virus isolation is considered the "gold standard" for virus identification [32], it can produce "false negative results" [20]. Virus isolation requires tissue heavily infected with intact virus [33], which is unlikely to be found in wild salmon which are culled by predators that target weakened fish [34]. As well, intact, infective ISAV may not reliably occur in healthy salmon that have been harvested for several days, such as fish found in markets. Molecular tests, however, have the capacity to detect low levels of virus fragments [35] making them ideally suited for the types of samples available to this study.

There is no universally accepted ISAV RT-qPCR TaqMan® assay. Most diagnostic laboratories use the Snow et al. [36] primer-probe set targeting a 104 bp-fragment on ISAV segment 8 [37, 38]. Some laboratories and researchers have found the Plarre et al. [31] primer-probe set targeting ISAV segment 7 to be more sensitive. Other researchers have published different ISAV segment 8 primer-probe sets that are highly sensitive [13], but are not included in the OIE Manual [38]. There is also a long standing conventional RT-PCR protocol targeting ISAV segment 8 using a primer set initially developed by Devold et al. [39], which is less sensitive than real time RT-qPCR. This yields a PCR product of 221 bp, which includes the first 94 bp of the 104 bp-PCR amplicon of the Snow et al. [36] RT-qPCR TaqMan® assay, with the reverse primer sequences of both assays overlapping in 9 nucleotides. While preliminary results from this study were interpreted as controversial [40, 41], they are consistent with the nature of both the tests and the samples, i.e., wild fish and fish from markets. The findings in the present study are supported by the many unpublished ISAV RT-PCR positive results in farmed and wild salmon in British Columbia, which exist as unpublished federal laboratory exhibits released by the Cohen Commission into the Decline of the Sockeye Salmon of the Fraser River [42]. Here we present more complete test results demonstrating that the ISAV sequences detected in British Columbia (BC) fish, both ISAV-HPRΔ and ISAV-HPR0, are of European genotype, with a mismatch in segment 8 that contributes to the inconsistent results of the RT-qPCR TaqMan® assay, and represents a new ISAV variant that appears to occur in BC in absence of high losses to the salmon farming industry. It would add to the knowledge of ISAV to test fresh moribund farmed salmon using the methods we describe here.

Results and Discussion

Sample RNA quality was based on real-time RT-PCR for ELF-1α as internal control for all samples

The ELF-1α controls showed a considerable variation between samples (within and between species). Fig. 1 highlights the systematic difference in the threshold cycle (C_t) values for the market-sampled vs. field-sampled fish. It also highlights two outliers in each of the two groups. The log sheets for the two anomalously large values in the field-sampled group indicate potential delays in sample processing under suboptimal conditions. The anomalously low values in the market-sampled group came from a single shopping event. This

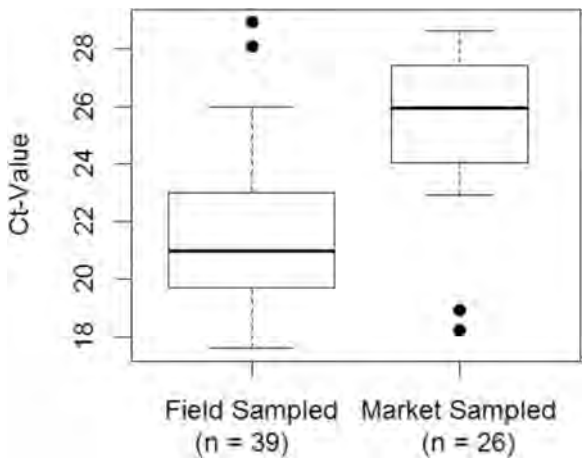


Fig. 1 Boxplots highlighting the systematic difference in the *Ct*-values for the market-sampled vs. field-sampled fish. The bold, middle line represents the median for each group; the box covers the range between the lower and upper quartiles; the whiskers extend to the last value still within 1.5 times the interquartile arrange of the relevant quartile; individual points beyond these are plotted individually as potential outliers.

in turn suggests that these fish were perhaps atypically fresh. Both these sets of outliers point to the potential for differences between sampling events to account for a substantial portion of the variability in these C_t values. A formal analysis of a mixed-effects model for these values using the R package, 'lme4' [43], provided the following estimates: (i) that the mean C_t values for the market-sampled group was 2.94 units higher than the mean for the field-sampled group, and (ii) that the standard deviation of the within-sampling-event means was 2.57, and that the residual deviation was only 1.47. Although the data set was too small to provide precise estimates of these parameters, it appears that most of the variability in the C_t values can be accounted for by variation in the sample quality – much of it unavoidably associated with the necessity to rely on (i) market purchases of farmed fish and (ii) logistics of sampling remote regions of British Columbia.

Fewer than 2.0 % of British Columbia fish tested were "non-negative" in the real time RT-qPCR TaqMan® assay for ISAV

In the present study, we used the Snow et al. [36] primer-probe set targeting segment 8 with a cut-off C_t value established as the mean C_t value in the highest virus dilution for which all 30 replicates were positive (Additional file 1: Table S1). Thus for the purposes of this study, samples were considered "non-negative" when the fluorescence signal increased above the C_t, and if the C_t value was ≤ 34.20. Samples with $C_t > 34.2$ to ≤ 39.9 were considered weak "non-negative" and > 40, suspicious as C_t of the last five cycles has higher uncertainty. Where the C_t value was zero, the result was deemed to be negative.

A total of 1,106 tissue samples were collected from market-sourced farmed salmon (397) and wild fish (708) obtained from (i) saltwater commercial fisheries, (ii) freshwater and saltwater sport fisheries, (iii) research sampling of juvenile salmon in seawater, and (iv) spawning adult salmon in freshwater habitat throughout British Columbia, Canada. As well, a sea louse (*Lepeophtheirus salmonis*) removed from a juvenile sockeye salmon was sampled. All samples were tested for ISAV using published real time RT-qPCR and/or conventional RT-PCR. The test results are detailed in Additional file 2: Table S2 and summarized in Table 2. Out of the total of 1,106 tissue samples, 20 (1.81 %) tested had a C_t value (Table **2**). Of these, only one sample (Fish # VR5, a spawning chum salmon in freshwater) was also positive in conventional RT-PCR with segment 8 and segment 6 HPR primers. One sample (Fish # TT48, an Atlantic salmon from a market) was also positive in conventional RT-PCR with segment 8, and two samples (Fish# SK20 and TT51, both Atlantic salmon from markets) were also positive in conventional RT-PCR with segment 6 HPR primers. The percentage of samples with a C_t value was more than two-fold greater in farmed fish tissues (2.77 %) compared to wild fish tissues (1.3 %). There were 56/65 fish that produced positive conventional RT-PCR results with no RT-PCR C_t values. All virus isolation attempts on these samples using ASK-2 and SHK-1 cell lines were negative. From a Canadian regulatory perspective, diagnostic confirmation requires virus isolation on permissive fish cell lines and virus identification [25] - hence a sample with a C_t value or positive conventional RT-PCR in this study was designated as "non-negative".

ISAV sequences detected in British Columbia fish have a mismatch in segment 8 compared to classical ISAV and represent a new ISAV variant of European genotype

Whereas all fish tissue samples were screened by the real time RT-qPCR TaqMan® assay for ISAV, only a portion of these samples was additionally tested by conventional RT-PCR for segment 8 or segment 6 HPR. Table 2 lists all non-negative test results by species and by farmed vs. wild status. This study did not attempt a direct comparison of the 3 different RT-PCR assays. Such an effort would require standardizing sample quality, which would require direct access to salmon in the farms.

To determine the genetic relationship between the ISAV sequences in this study and ISAV strains worldwide, we compared the segment 8 sequences using multiple alignment and phylogenetic analysis. All the 50 sequences from this study aligned well in a 221 bp-long fragment with 47 selected classical ISAV segment 8 sequences of different ISAV isolates in GenBank (GenBank Database) (Fig. 2). This alignment revealed a consistent single nucleotide mutation (5'-CAT CGT CGC TGC AGA TC-3') in the 3' region of the probe sequence (5'-CAT CGT CGC TGC AGT TC-3') [36]. This mutation in the BC samples would contribute to the apparent failure of the real time RT-qPCR TaqMan® assay for ISAV in the 49 samples, positive in segment 8, but with no C_t value in the real time RT-qPCR TaqMan® ISAV assay. None of the classical ISAV segment 8 sequences in the GenBank database have this mutation. While a single nucleotide variation is a minor mutation, its placement in a region that an ISAV probe sequence seeks to anneal in a standard OIE ISAV test, makes this a significant mutation that warrants recognition as a new variant. Improvements aimed at better detection of this variant are currently being developed. Recognition of, and testing for, this variant are essential prerequisites for determining how widespread it is.

The alignment of amino acid sequences in a 118 bp-long fragment of the same ISAV sequences in Fig. 2 without the primer sequences is shown in Fig. 3. The polypeptide aligned well. The number of mutations shown in Fig. 3 is slightly less than the number of

Table 2 Number of samples for each species that (i) tested non-negative for infectious salmon anaemia virus (ISAV) by RT-qPCR[1] and that produced sequences by conventional PCR for (ii) segment 6 and (iii) segment 8

Fish species		Farmed fish				Wild fish			
Common name	Scientific name	n	RT-qPCR	Conventional PCR seg. 8 sequence	Conventional PCR seg. 6 sequence	n	RT-qPCR	Conventional PCR seg. 8 sequence	Conventional PCR seg. 6 sequence
Atlantic salmon	*Salmo salar*	334	9	18	13[2]				
Chinook salmon	*Oncorhynchus tshawytscha*	13	0	0	0	102	2	2	0
Coho salmon	*Oncorhynchus kisutch*	4	0	0	0	68	1	6	1
Sockeye salmon	*Oncorhynchus nerka*					256	3	7	2
Kokanee	*Oncorhynchus nerka*					1	1	1	Na
Pink salmon	*Oncorhynchus gorbuscha*					118	0	2	0
Chum salmon	*Oncorhynchus keta*					68	1	1	1
Steelhead trout	*Oncorhynchus mykiss*	46	2	3	0	21	0	Na	Na
Cutthroat trout	*Oncorhynchus clarkii*					18	0	8[3]	13
Chum mackerel	*Scomber japonicus*					13	0	1	0
Pacific herring	*Clupea pallasi*					44	0	1	0
Sea louse	*Lepeophtheirus salmonis*					1	1	0	0
Total		397	11	21	13	709	9	29	17

[1]All fish tissue samples were screened by the real time RT-qPCR TaqMan® assay for ISAV. However, only some of the samples were also tested in conventional RT-PCR for segment 6 HPR or segment 8. Therefore, the numbers do not reflect a direct comparison of the 3 different RT-PCR assays. Na denotes Not applicable.
[2]2 others with a PCR product but not sequenced.
[3]5 others with a PCR product but not sequenced.

mutations in Fig. 2. This is because of the redundancy nature of the genetic code. For example, the mutation from AAAGCCC to AAGGCCC in VT02142014-120 is not shown in Fig. 3; this is because both AAA and AAG are translated to K. The single nucleotide mutation in the probe sequence of Snow et al. [36] resulted in a single amino acid change from V to D and would therefore produce a functional full-length viral protein.

Phylogenetic analysis was used to further determine the genetic relationship between the newly discovered BC ISAV variant in the present study and the classical ISAV strains worldwide. Fig. 4 shows the phylogenetic tree generated with these sequences with satisfactory bootstrap support (bootstrapping values more than 70 % are marked). In addition to showing the relationship of the ISAV sequences from this study (all "VT" sequences), this tree also supports the well-established major division between North American genotype and European genotype ISAV. All the ISAV sequences detected in this study are of the European genotype. While these BC sequences tend to be similar to each other, their differences with other European sequences are very small. The apparent wide diversity of the BC sequences in the tree reflects the nature of the sampling, and the fact that there was no single-source-selected amplification as occurs with the classical ISAV isolates from disease outbreaks. Moreover, the branching in the tree also indicates that some (VT02142012-120, VT08092012-449, and VT08092012-402) diverted early from most of the classical European genotype sequences, while others (VT06062013-60, VT08092012-465, VT05012012-308, and VT06202012-391) have evolved separately. The data support the observation that ISAV can exist in a region for a period of years in absence of outbreaks and in a state that may not be detectable by methods designed to diagnose virulent outbreaks in moribund farmed fish.

ISAV sequences detected in British Columbia fish include both ISAV-HPRΔ and ISAV-HPR0 and are of European genotype

The detection of ISAV-HPR0 in British Columbia fish (Fish# SK20) was designated a suspect result by the

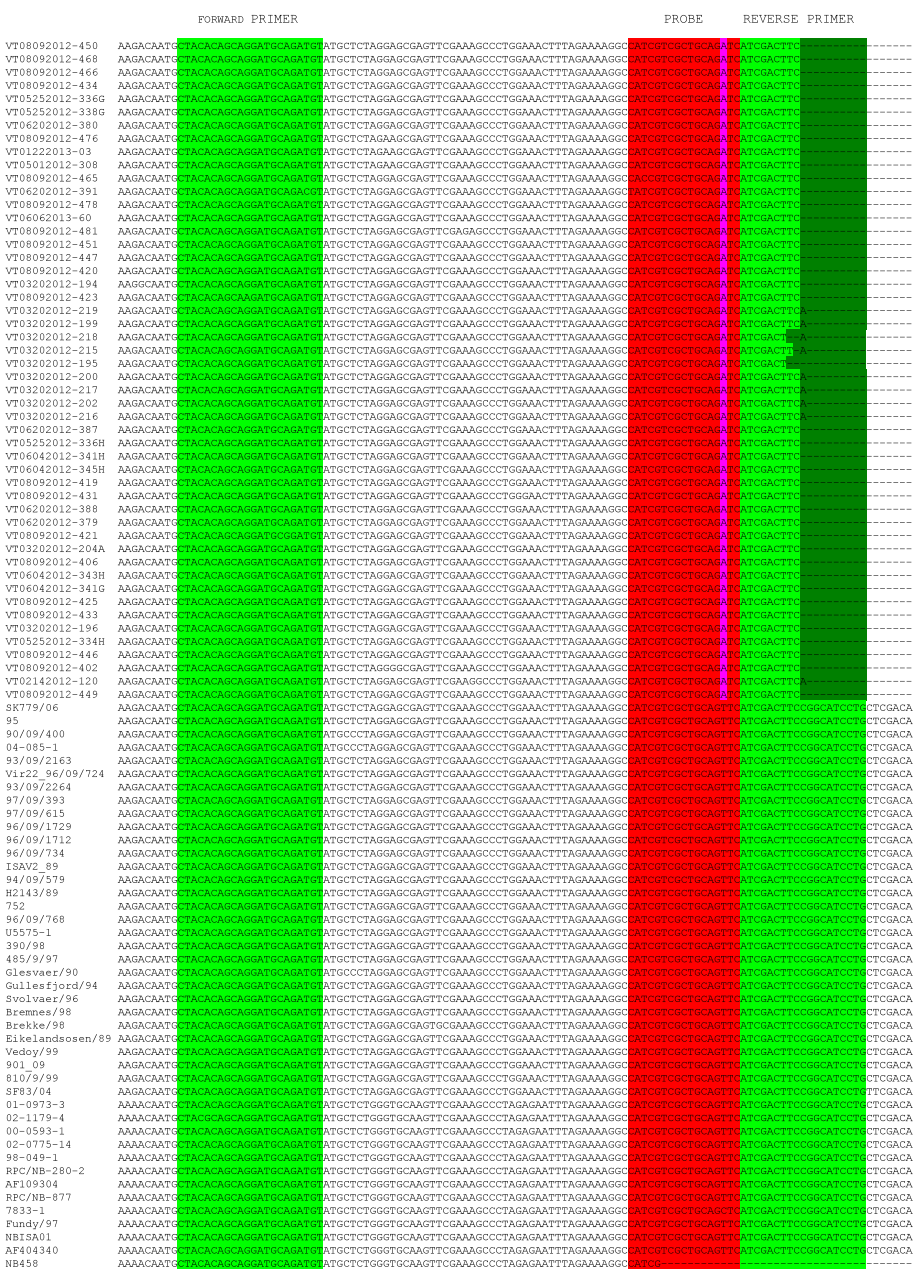


Fig. 2 Alignment of ISAV segment 8 nucleotide sequences in the target region of the primer-probe set of the real time RT-qPCR TaqMan® assay for ISAV [36]. The ISAV sequences belonged to a 221 bp-long PCR product amplified from 50 samples in this study and 47 selected classical ISAV segment 8 sequences of different ISAV isolates in the GenBank database. The nucleotide sequences were aligned using CLUSTAL X with the default settings [58]. Green is forward primer and reverse primer sequences and red is probe sequence. The single nucleotide mutation in the probe sequence is in blue. The reverse primer sequence includes the 9-nucleotide overlap between the reverse primer sequence of the real time RT-qPCR TaqMan® assay [36] and that of the conventional RT-PCR protocol [39]

Canadian Food Inspection Agency (CFIA), because of the inability for follow up by the federal authorities. With the widespread occurrence of ISAV-HPR0 variants in many parts of the world and its potential as a precursor to the virulent strains of ISAV [13], it is essential that RT-PCR positive results based on segment 8 primers be followed up with conventional RT-PCR using segment 6 primers targeting the HPR. Sequencing of the PCR product is also essential in order to determine the ISAV HPR type present (ISAV-HPRΔ or ISAV-HPR0 or both) [9]. ISAV-HPR0 has only been reported in apparently healthy fish and has never been associated with clinical or pathological diagnosis of ISA disease [44].

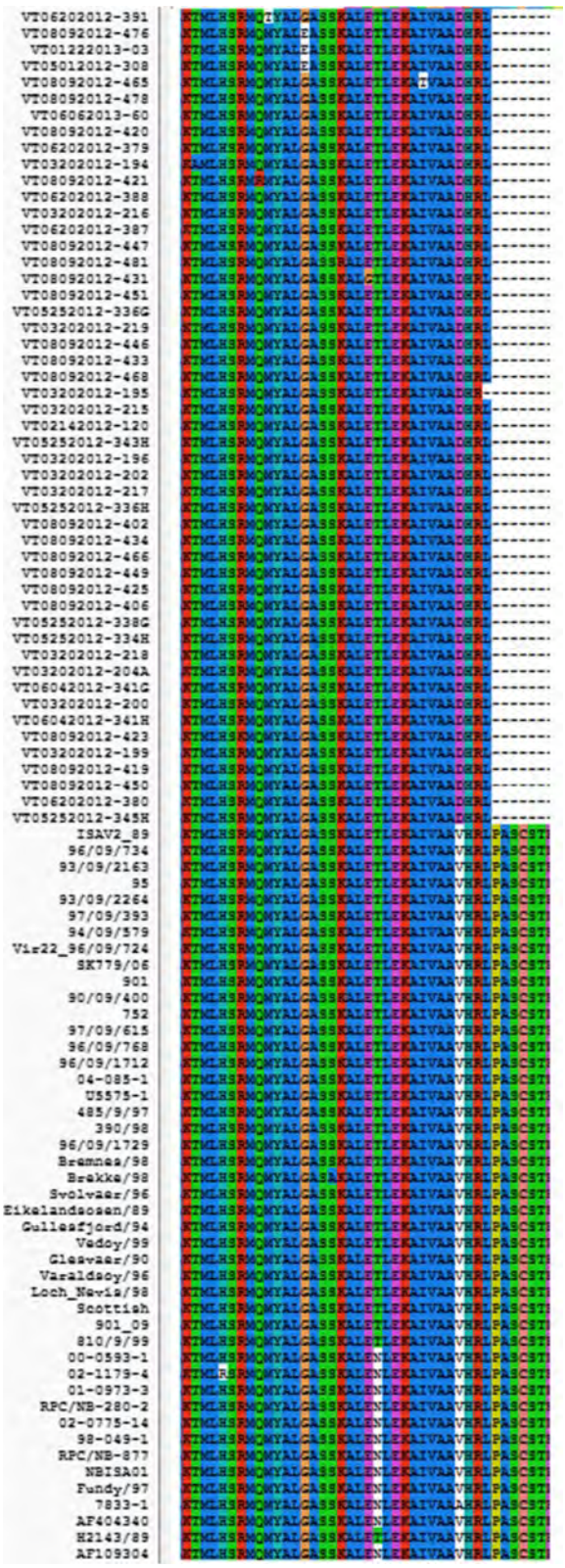


Fig. 3 Alignment of ISAV segment 8 amino acid sequences in the target region of the primer-probe set of the real time RT-qPCR TaqMan® assay for ISAV [36]. The ISAV sequences are those in Fig. 2. The amino acid sequences in the region between the two primers (Forward Primer and Reverse Primer) were aligned using CLUSTAL X with the default settings [58]. The single nucleotide mutation in the probe sequence resulted in a single amino acid change from V to D

Of the fish tested in conventional RT-PCR for segment 6 HPR, sequences of the PCR product were obtained from 13 farmed fish samples (13 Atlantic salmon) and 17 from wild fish samples (1 coho, 2 Sockeye, 1 Chum and 13 Cutthroat) (Table 2). In contrast to conventional RT-PCR for segment 8, where 49 samples positive in segment 8 had no C_t value in the real time RT-qPCR TaqMan® assay for ISAV, only 3 samples (Fish# SS132, MQ06, and P113, Additional file 2: Table S2) were positive in conventional RT-PCR for segment 6, with no C_t value. These samples were also negative by conventional RT-PCR for segment 8.

The sequences of ISAV segment 6 obtained from the PCR products matched ISAV-HPR5 on 29 samples, one had both ISAV-HPR5 and ISAV-HPR7b and one sample matched ISAV-HPR0 (Additional file 2: Table S2). All were of European genotype. ISAV-HPRΔ strains of HPR5 and HPR7b types have been associated with ISA outbreaks in Norway [45, 46], Scotland [47] and Chile [9, 48]. Thus our data indicate that ISAV-HPRΔ strains can be present without clinical disease in farmed fish and without being detected by virus isolation, which is in agreement with other reports [15, 16].

To determine the genetic relationship between the segment 6 HPR sequences detected in BC and worldwide, we compared the HPR sequences using multiple alignment and phylogenetic analysis. A total of 316 sequences aligned well and the phylogenetic tree was generated depicting the overall relationship among all ISAV isolates for which segment 6 sequence is available (data not shown). Virulent ISAV isolates have a deletion in segment 6 HPR sequence [44]. Of the 316 segment 6 HPR sequences, we only identified 101 sequences that were long enough to display this deletion. We aligned these 101 sequences to show the deletion. No existing alignment software packages can align nucleotide sequences in this delicate and complex area, thus the alignment has been manually adjusted. When we prepared a figure to show these sequences and the deletion, we found it is hard to show so many sequences in a figure so that some of the sequences that behave the same in this deletion area were removed. Fig. 5 shows a portion of the alignment containing the deletion. In this figure, 71 sequences were included, representing the 101 sequences. The first five sequences of the alignment, including VT12212012-1068, are complete, i.e., they have

Fig. 4 Phylogenetic tree showing the genetic relationship between the 97 ISAV sequences aligned in Fig. 2. Sequences were aligned and the phylogenetic tree was generated by using CLUSTAL X with the default settings [58]. Phylogenetic analysis using Neighbor-Joining bootstrap method (1000 replicates) provided satisfactory bootstrap support (bootstrapping values are shown for branch-points with greater than 70 % bootstrap support)

no deletion and belong to ISAV-HPR0 [49]. The rest of the sequences have a deletion and belong to ISAV-HPRΔ [11, 44].

The alignment of amino acid sequences of the ISAV segment 6 HPR sequences in Fig. 5 without the primer sequences is shown in Fig. 6. Both figures show extensive deletions in the HPR. Existing software cannot produce high-quality alignments in areas with deletions. Thus this alignment was manually adjusted to reveal the deletion event. The deletions in HPR probably occur through homologous recombination (copy-choice recombination, presumably because of strand-switching by the viral RNA polymerase [50] during negative RNA strand synthesis from one nucleic acid template of one virus to another. Fig. 6 confirms the three ISAV HPR types found in the BC samples: ISAV-HPR0 (VT12212012-1068), ISAV-HPR7b (VT03282012-222-C and VT02142012-90), and ISAV-HPR5 (VT05252012-337G and 5 other samples) and also reveals the relationship

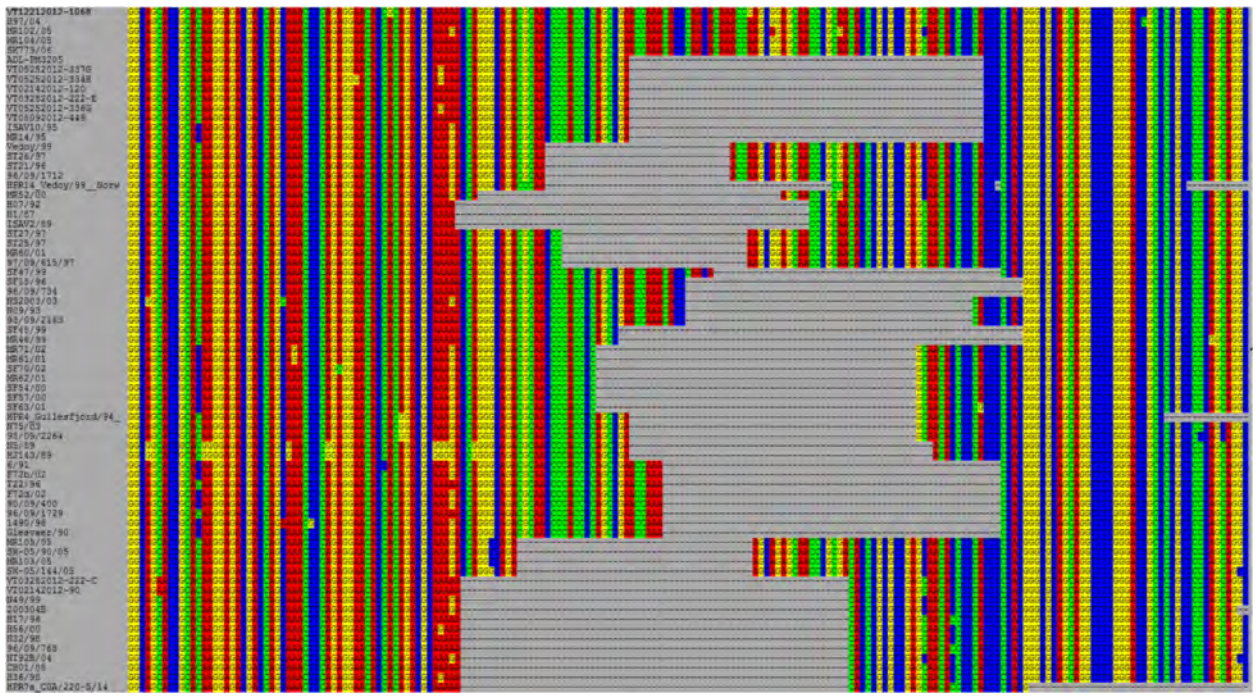


Fig. 5 Alignment of ISAV segment 6 HPR nucleotide sequences containing the deletion. The alignment was adjusted manually as all known alignment software packages cannot align nucleotide sequences in such a delicate and complex area. Among 101 sequences analyzed, 39 of them are British Columbia isolates (ID starts with VT); the deletions of these 39 sequences are in three patterns. The first pattern is VT12212012-1068; the second pattern is VT03282012-222-C and VT02142012-90; the third pattern has 36 isolates and this pattern can be shown in VT05252012-337G. To save space in the figure, we used 6 sequences to represent the 36 sequences in this pattern

between these HPR types and ISAV HPR types in other countries.

Fig. 7 shows the phylogenetic tree generated with the sequences in Fig. 4 with satisfactory bootstrap support (bootstrapping values more than 70 % are marked); where NBISA01/98 is used as an outgroup. This tree contains only the isolates from Fig. 5. It was not created to reflect the whole evolutionary history of ISAV segment 6, but to help with the analysis of the HPR deletion. Comparing Figs. 5, 6 and 7, we can find some consistency, i.e. isolates that show the same deletion pattern tend to be closer inside the tree. Although such consistency only exists for a few groups, the combination of these two approaches may reveal more insights on ISAV's evolutionary history.

Conclusions

To our knowledge the present work constitutes the first published report of the detection of ISAV sequences in fish from British Columbia, Canada. The sequences detected, both of ISAV-HPRΔ and ISAV-HPR0 are of European genotype. The virus in these samples has a mismatch in segment 8 that can account for failure of the real time RT-qPCR TaqMan® assay for ISAV recommended in the OIE Aquatic Manual. Furthermore, these sequences are different from the classical ISAV segment 8 sequences, and this difference suggests the presence of a new ISAV variant of European genotype in BC. Our results further suggest that ISAV-HPRΔ strains can be present without clinical disease in farmed fish and without being detected by virus isolation using fish cell lines. Recent reports on ISAV surveillance in Washington, USA [41], and in British Columbia [17] report no ISAV detection. However, neither of these studies report on samples from the known target host of ISAV, farmed Atlantic salmon, and it is unreported whether weak RT-PCR positives similar to ours were found, and interpreted as "negative". More research on the source of this variant ISAV sequence is critically important for assessing the risks to both farmed and wild salmon in the region, its origin and to better understand ISAV evolution.

Methods

Sampling

Wild fish were collected from freshwater spawning grounds, fresh and saltwater sport fisheries, saltwater commercial fisheries, and saltwater scientific fisheries. Wild fish samples included all species of Pacific salmon (*Oncorhynchus* sp.), Atlantic salmon (*Salmo salar*), steelhead (*Oncorhynchus mykiss*), cutthroat trout (*Oncorhynchus clarkii*), kokanee (*Oncorhynchus nerka*), Pacific chub mackerel (*Scomber japonicus*) and Pacific herring (*Clupea*

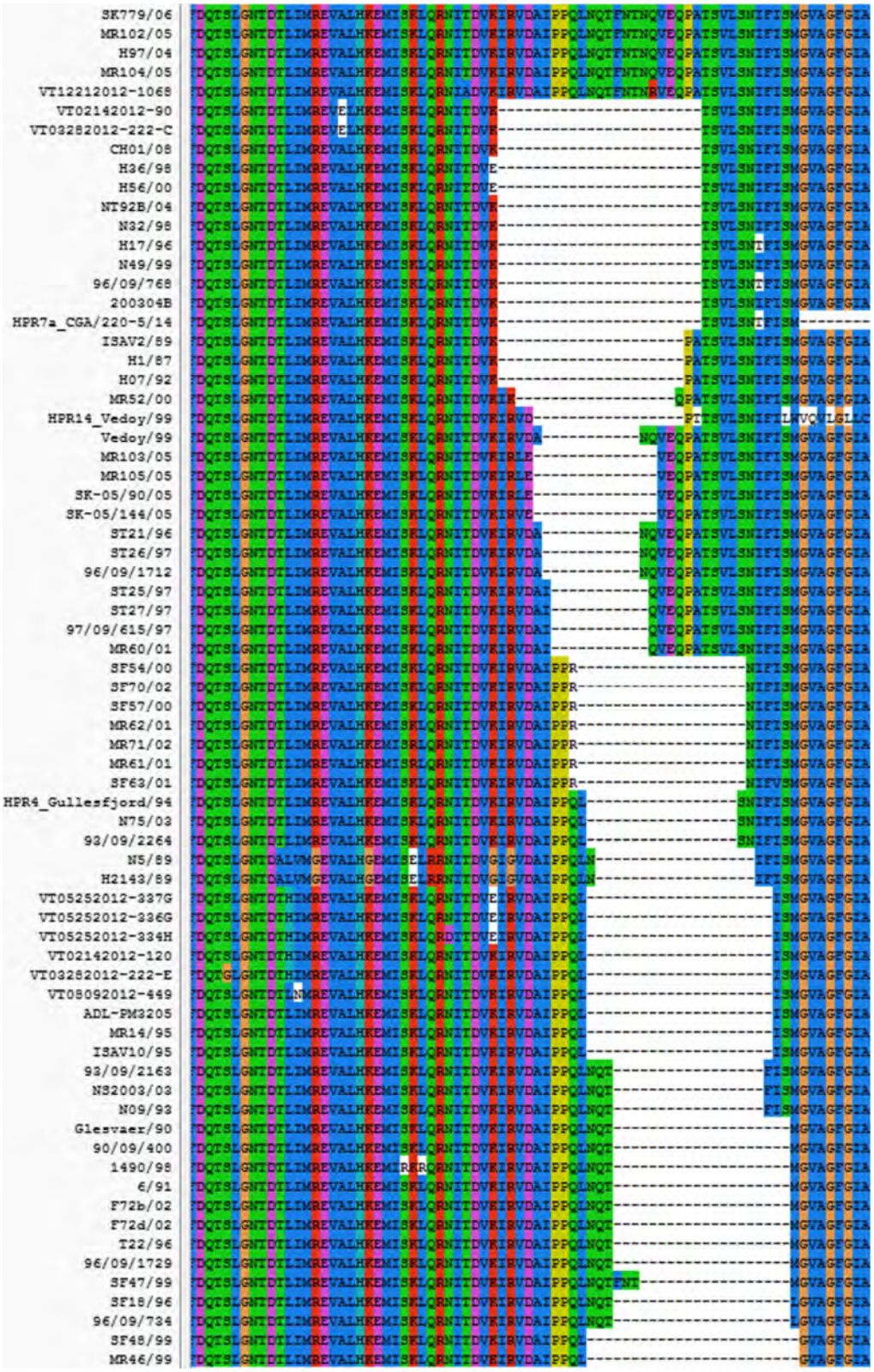


Fig. 6 (See legend on next page.)

(See figure on previous page.)
Fig. 6 Alignment of ISAV segment 6 HPR amino acid sequences containing the deletion. The alignment was adjusted manually as all known alignment software packages cannot align amino acid sequences in such a delicate and complex area in Fig. 5. The alignment confirms the three ISAV HPR patterns in Fig. 5: The first pattern VT12212012-1068 belongs to ISAV-HPR0; the second pattern VT03282012-222-C and VT02142012-90 belongs to ISAV-HPR7b; the third pattern with 36 isolates and shown in VT05252012-337G belongs to ISAV-HPR5. Similarly to Fig. 5, only 6 of the 36 sequences in the third pattern are included in Fig. 6

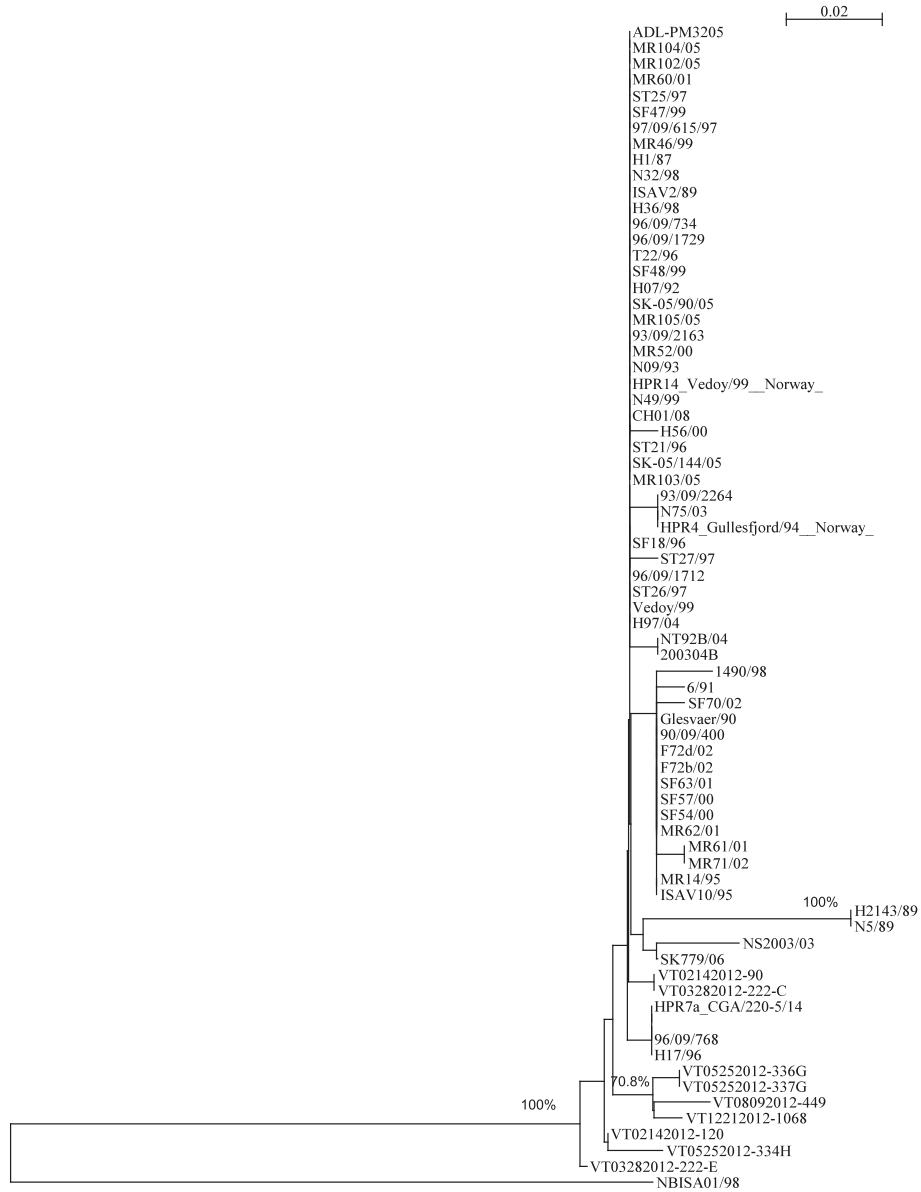


Fig. 7 Phylogenetic tree showing the genetic relationship between the 71 representative segment 6 HPR sequences aligned in Fig. 4. Sequences were aligned and the phylogenetic tree was generated by using CLUSTAL X with the default settings [58]. Phylogenetic analysis using Neighbor-Joining bootstrap method (1000 replicates) provided satisfactory bootstrap support (bootstrapping values are shown for branch-points with greater than 70 % bootstrap support)

pallasi) (Table 2). All fish and the sampled organs were photographed *in situ*. Gill and heart were sampled from the whole wild fish. Gill and remnant head kidney were sampled from the gutted, head-on farmed salmon and also the farmed salmon heads purchased from markets. The hearts were not available from these samples. All samples were placed immediately in sterile Whirl-Pak® bags (Nasco Inc., Fort Atkinson, WI) on ice with replicate samples preserved in RNALater® (Ambion Inc., Foster City, CA) and shipped overnight by courier to the testing laboratory. At the laboratory, samples were immediately stored at −80 °C until they were analyzed. The testing laboratory ran tests exclusively on the samples and did not participate in the collection of the samples or in the custody of the samples prior to receipt of the samples.

Total RNA preparation

Total RNA was isolated using a modified total RNA extraction protocol with the RNeasy® mini Kit (QIAGEN). Briefly, each tissue (or pool of tissues) was weighed and macerated to a 10 % suspension w/v in phosphate buffered saline (PBS) with 10x antibiotics. The specimen supernatant was used for RNA extraction. Samples preserved in RNAlater® were first washed three times with PBS and then homogenized as described above prior to total RNA extraction. Total RNA was isolated from samples using 1.25 ml of TRIZOL Reagent (Invitrogen) and 375 μl of sample volume as previously described [51]. The Viral RNA mini Kit (QIAGEN) was also utilized on selected samples following the manufacturer's recommended protocol. In all cases, the extracted RNA was eluted in 20–50 μl of nuclease-free water, and RNA yields were quantified and purity analysed using the OD260/280 ratio and a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The eluted RNA was tested immediately following quantitation, or was stored frozen at −80 °C prior to use in RT-PCR.

Real-time RT-qPCR

RT-qPCR was run on the LightCycler 480 (Roche Applied Science), version 4.0. The crossing point (Cp) or threshold cycle (C_t) was determined by use of the maximum-second-derivative function on the LightCycler software release 1.5.0. The Roche LightCycler® 480 RNA master Hydrolysis Probe kit (Roche Diagnostics) was employed for all RT-qPCR reactions according to the manufacturer's specifications. Sample RNA quality was based on RT-qPCR for elongation factor 1 alpha (ELF-1α) as internal control targeting either Atlantic salmon ELF-1α (GenBank accession number BT072490) or Chinook salmon ELF-1α (GenBank accession number FJ890356) using primers, probes, and RT-qPCR thermal cycling parameters as previously reported [52]. RNA quality varied, with the higher C_t values generally occurring in farmed salmon from markets where the interval between harvest and sampling was on the order of days, not minutes as was the case for most wild fish samples. Nonetheless, some wild fish, such as the cutthroat trout and LaP1, were caught by fishermen with unavoidable delays in processing the sampled fish. Such delays may have contributed to the higher C_t values in some of these samples. Results from tests with C_t values above 40 or at 0 were designated as negative. In addition, these samples would be considered unfit for further testing if after re-extraction and repeated RT-qPCR the same results were obtained.

Detection of ISAV with the one-step real-time RT-qPCR [51] was carried out using the primer-probe set developed by Snow et al. [36] targeting segment 8 and described in the OIE Aquatic Manual [38]. However, there is no defined C_t value cut off to aid interpretation of results. In this study, the cut-off C_t value for this probe was set at $\leq 34.20 \pm 1.05$ based on 10-fold dilutions of cell culture ISAV (ADL 2007) each tested in 5 replicates and repeated 6 times for a total of 30 replicates, and denotes the mean C_t value in the highest virus dilution for which all 30 replicates were positive (Additional file 1: Table S1). The same preparations were also tested in the conventional RT-PCR methods below, allowing for a correlation of the cut-off C_t value with the conventional RT-PCR tests on cell culture virus.

Conventional RT-PCR and nucleic acid sequencing

Samples testing positive by real-time RT-qPCR were further tested using conventional one step RT-PCR targeting segments 6 and 8 to obtain PCR products for DNA sequencing. The ISAV conventional one step RT-PCR used ISAV-specific primers FA-3/RA-3 and the conditions described by Devold et al. [39] for RNA segment 8, and the following primers segment 6 HPR primers, Fwd 5'-GCC CAG ACA TTG ACT GGA GTA G-3', and Rev 5'-AGA CAG GTT CGA TGG TGG AA-3' described by Kibenge et al. [9] for RNA segment 6, and was run in a Bio-Rad thermal cycler (Bio-Rad). Briefly, amplification was performed using 50 μl reaction mixture utilizing One-step RT-PCR kit (QIAGEN) as follows: the reaction mixture contained 2 μl of total RNA, 10 μl of 5X QIAGEN OneStep RT-PCR buffer, 2 μl of dNTPs, 10 units of RNAse inhibitor (Life Technologies), 0.6 μM (final concentration) of each primer pair, and 2 μl of QIAGEN OneStep RT-PCR enzyme mix in a final volume of 50 μl. The RT-PCR amplification conditions were 1 cycle at 50 °C for 30 min, one cycle at 95 °C for 15 min, 40 cycles at 94 °C for 30 s, 60 °C for 60 s and 72 °C for 90 s and 1 cycle at 72 °C for 10 min before soaking at 4 °C. Amplified products were analyzed by electrophoresis on 1 % agarose gel and purified using High Pure PCR Product

Table 3 GenBank Accession numbers used in the multiple alignments and phylogenetic analyses and of new sequences from this study

Isolate or sample ID	Segment 6	Segment 8	Reference
VT02142012-120	JQ857081	JQ857078	This study
VT05252012-334H	KR998473	KR998431	This study
VT05252012-336G	KR998474	KR998432	This study
VT08092012-449	KR998475	KR998433	This study
ISAV2/89	DQ785246		
96/09/734	DQ785250	DQ785278	
96/09/768	DQ785249	DQ785277	
96/09/1729	DQ785251	DQ785279	
96/09/1712	DQ785245	DQ785273	
93/09/2264	DQ785255	DQ785283	
93/09/2163	AF427049	DQ785281	
HPR4_Gullesfjord/94_Norway	AF302801	AF262384	
HPR14_Vedoy/99_Norway	AF302803	AF262383	
Glesvaer/90	AF220607	AF262382	
90/09/400	DQ785248	DQ785276	
H2143/89	DQ785247	DQ785275	
SK779/06	EU118820	EU118822	
NBISA01/98	AF283996	AF315063	
VT03282012-222-C	KR998476		This study
VT03282012-222-E	KR998477		This study
VT05252012-337G	KR998478		This study
VT12212012-1068	KR998479		This study
ADL-PM3205	HQ011267		
MR104/05	DQ108607		
MR102/05	DQ108605		
MR60/01	AY127876		
ST25/97	AF364885		
SF47/99	AF364888		
97/09/615/97	DQ785252		
MR46/99	AF364896		
HI/87	AF364893		
N32/98	AF364883		
H36/98	AF302799		
T22/96	AF364889		
SF48/99	AF364878		
H07/92	AF364898		
SK-05/90/05	FM203287		
MR105/05	DQ108608		
MR52/00	AF364892		
N09/93	AF364895		
N49/99	AF364876		
CH01/08	EU851043		
H56/00	AF364880		
ST21/96	AF364886		
SK-05/144/05	FM203274		
MR103/05	DQ108606		
N75/03	AY971661		
SF18/96	AF364869		
ST27/97	AF364897		
ST26/97	AF364879		
H97/04	DQ108604		
NT92B/04	AY973188		
200304B	FM203244		
1490/98	AF391126		
6/91	AF364894		
SF70/02	AY127880		
F72d/02	AY971657		
F72b/02	AY971656		
SF63/01	AY127879		
SF57/00	AF364890		
SF54/00	AF364884		
MR62/01	AY127878		
MR61/01	AY127877		
MR71/02	AY127881		
MR14/95	AF364873		
ISAV10/95	DQ785254		
N5/89	AY127882		
NS2003/03	AY973182		
HPR7a_CGA/220-5/14	KJ944288		
96/09/768	DQ785249		
H17/96	AF364891		
VT03202012-194		KR998434	This study
VT03202012-195		KR998435	This study
VT03202012-196		KR998436	This study
VT03202012-199		KR998437	This study
VT03202012-200		KR998438	This study
VT03202012-202		KR998439	This study
VT03202012-204A		KR998440	This study
VT03202012-215		KR998441	This study
VT03202012-216		KR998442	This study
VT03202012-217		KR998443	This study
VT03202012-218		KR998444	This study
VT03202012-219		KR998445	This study

Table 3 GenBank Accession numbers used in the multiple alignments and phylogenetic analyses and of new sequences from this study *(Continued)*

VT05012012-308	KR998424	This study
VT05252012-336H	KR998425	This study
VT05252012-338G	KR998446	This study
VT06042012-341G	KR998447	This study
VT06042012-341H	KR998448	This study
VT06042012-343H	KR998449	This study
VT06042012-345H	KR998450	This study
VT08092012-402	KR998451	This study
VT08092012-406	KR998452	This study
VT08092012-419	KR998453	This study
VT08092012-420	KR998454	This study
VT08092012-421	KR998455	This study
VT08092012-423	KR998456	This study
VT08092012-425	KR998457	This study
VT08092012-431	KR998426	This study
VT08092012-433	KR998427	This study
VT08092012-434	KR998458	This study
VT08092012-446	KR998428	This study
VT08092012-447	KR998459	This study
VT08092012-450	KR998460	This study
VT08092012-451	KR998461	This study
VT08092012-465	KR998462	This study
VT08092012-466	KR998463	This study
VT08092012-468	KR998464	This study
VT08092012-476	KR998465	This study
VT08092012-478	KR998466	This study
VT08092012-481	KR998467	This study
VT06202012-379	KR998468	This study
VT06202012-380	KR998469	This study
VT06202012-387	KR998470	This study
VT06202012-388	KR998429	This study
VT06202012-391	KR998471	This study
VT01222013-03	KR998430	This study
VT06062013-60	KR998472	This study
Vir22_96/09/724	DQ785286	
97/09/393	DQ785284	
97/09/615	DQ785280	
94/09/579	DQ785285	
SF83/04	AY744395	
Svolvaer/96	AF262381	
Bremnes/98	AF262385	
Brekke/98	AF262380	

Table 3 GenBank Accession numbers used in the multiple alignments and phylogenetic analyses and of new sequences from this study *(Continued)*

Eikelandsosen/89	AF262386
810/9/99	DQ022085
95	DQ785282
901	GU830910
752	GU830902
390/98	DQ003602
485/9/97	DQ003605
U5575-1	DQ003603
04-085-1	DQ058660
01-0973-3	DQ003607
02-1179-4	DQ003601
00-0593-1	DQ003606
02-0775-14	DQ003604
98-049-1	DQ003600
RPC/NB-280-2	AF312317
AF109304	AF109304
RPC/NB-877	AF312316
7833-1	AF312315
Fundy/97	AF262389
AF404340	AF404340
NB458	AY151798

Purification Kit (Roche). The PCR products were cloned into the pCRII vector using a TOPO TA cloning kit (Invitrogen) in preparation for nucleotide sequencing, although in some cases the RT-PCR products were sequenced directly without cloning. Plasmid DNA for sequencing was prepared as per Kibenge et al. [53], and DNA sequencing as per Kibenge et al. [10] by ACGT Corporation (Toronto, Ontario, Canada). Sequence analysis used the BLAST programs [54] against the latest release at GenBank [55], the Sequence Manipulation suite version 2 [56], and the FASTA program package for microcomputers [57]. Sequences are available through GenBank and their accession numbers are listed in Table 3.

Phylogenetic analyses

Sequences were aligned and phylogenetic trees were generated using CLUSTAL X with the default settings [58]. Alignment regions containing gaps were excluded from the analysis. The results were analyzed by using the bootstrap method (1000 replicates) to provide confidence levels for the tree topology. We then used different outgroup sequences to determine and verify the root of each tree.

Virus isolation

Primary virus isolation was attempted on some of the RT-PCR "non-negative" samples using Salmon head kidney (SHK-1 and ASK-2) cell line monolayers. SHK-1 [59] and ASK-2 cells [39] were grown as previously described [12]. Homogenized tissues were inoculated on monolayers of SHK-1 and/or ASK-2 cell lines following standard protocols in the OIE Aquatic Manual [38]. Briefly, each tissue was weighed and macerated to a 10 % homogenate w/v in PBS with 10x antibiotics. The homogenates were centrifuged at 205.3 g for 15 min at 4 °C. The supernatants were individually filtered using 0.45 μM syringe filters to remove any bacteria prior to use in virus isolation attempts. 24 hr-old cell monolayers in tissue culture flasks free of medium were inoculated with the sample supernatant diluted 1:10 in serum-free medium, and incubated for 2 hr at room temperature to allow for virus adsorption. Maintenance medium was then added and the inoculated cells were then incubated at 16 °C and infection was allowed to proceed with daily monitoring using an inverted light microscope until the CPE was evident or 21 days and the flasks were frozen at −80 °C. Virus isolation was monitored by RT-PCR on the cell lysates since virus replication may occur without development of apparent CPE [60]. CPE negative and RT-PCR negative cultures were passaged on fresh cell monolayers. A sample was considered negative if no CPE or positive RT-PCR was observed after three blind passages.

Ethics

The *in vitro* work was approved by the UPEI Biosafety Committee.

Additional files

Additional file 1: Correlation of mean Ct value with conventional RT-PCR with ISAV segment 8 and HPR primers on cell culture virus. Table showing Correlation of mean C_t value with conventional RT-PCR with ISAV segment 8 and HPR primers on cell culture virus. (DOC 33 kb)

Additional file 2: Fish tissue samples testing "non-negative" for infectious salmon anaemia virus (ISAV) from 2012-2013[1]. Table listing the ISAV segment 8 tests done in replicates, which were in some cases repeated. Values in this column represent how many replicates produced a C_t value, or the averaged result of tests. (DOC 117 kb)

Competing interest

The authors declare they have no competing financial interest in relation to this report.

Authors' contributions

MJTK isolated total RNA from tissue samples, performed the RT-qPCR for ELF-1α and ISAV, conventional RT-PCR for ISAV, cloned PCR products for sequencing, and helped to write the manuscript. TI performed conventional RT-PCR for ISAV and cloned PCR products for sequencing and helped to write the manuscript. YW performed all the multiple alignments and phylogenetic analyses and helped to write the manuscript. AM provided the Canadian samples for diagnostic testing and helped to write the manuscript. RR provided the Canadian samples for diagnostic testing, performed the statistical analysis of the ELF-1α C_t values and helped to write the manuscript. FSBK performed the virus isolation attempts, coordinated all viral testing and DNA sequence analysis and helped to write the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the Virology Research Laboratory at the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, PE, Canada, and a Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery Grant to F.S.B.K. Van City, Rudy North, James Corbett, Patagonia, Tony and Kim Allard, Dick and Val Bradshaw, Vancouver Foundation, the Tula Foundation, Elizabeth Borak, Wheeler Family Foundation, Yvon Chouinard, Eden Conservation Trust, Sarah Haney, Tides Foundation, Jaymac Foundation, an NSERC grant to R.R. and many smaller donors. Thank you to the First Nations who assisted and allowed us to sample their fish - Boston Bar, Gitanyow Fisheries, Gitxsan Fisheries Authorities, Heiltsuk, Kwikwasut'inuxw Haxwa'mis, Lake Babine Nation Fisheries, Lake Cowichan, Mowachaht/Muchalaht, N'Quatqua, Nicola, Seton Lake, Shuswap, Skeena First Nations, Snuneymuxw, Splatsin, St'at'imc, Stellaquo, Stó:lô , Takla Lake, Xeni Gwet'in, Office of the Wet'suwet'en Fisheries and Wuikinuxv First Nations. We also thank Rob, Nola, Krystal and Amber-Bachen, Sandy Bodrug, Farlyn and Tavish Campbell, Tamara and Roy Campbell, Brad Crowther, Roger Dunlop, Jody Eriksson, Randy Ericksen, Dave and Nicole Gerbrandt, Nicole, Donna and Bill Mackay, Jennifer Parkhouse, Stan Proboszcz, Anissa Reed, Dave Rolston, Louise Routledge, Steve Sharron, Tsylos Park Lodge, Ted and Duane Walkus, Monica Woods, and Sabra Woodworth.

Author details

[1]Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, P.E.I. C1A 4P3, Canada. [2]Department of Computer Science, University of Prince Edward Island, 550 University Ave., Charlottetown, P.E.I. C1A 4P3, Canada. [3]Raincoast Research Society, Box 399, 390 1st Street, Sointula, BC V0N 3E0, Canada. [4]Department of Statistics and Actuarial Science, Simon Fraser University, 8888 University Drive, Burnaby, B.C. V5A 1S6, Canada. [5]Current address: Diagnostic Services Unit, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, P.E.I. C1A 4P3, Canada.

Received: 27 October 2015 Accepted: 28 December 2015
Published online: 06 January 2016

References

1. McCauley J, Hongo S, Kaverin N, Kochs G, Lamb R, Matrosovich M, et al. Family *Orthomyxoviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. San Diego, CA: Academic; 2011. p. 749–61.
2. Adams S, Xing Z, Li J, Kristelle M, Perez D, Reed K, et al. The effect of avian influenza virus NS1 allele on virus replication and innate gene expression in avian cells. Mol Immunol. 2013;56:358–68.
3. Hause B, Collin E, Liu R, Huang B, Sheng Z, Lu W, et al. Characterization of a novel influenza virus strain in cattle and swine: proposal for a new genus in the *Orthomyxoviridae* family. MBio. 2014;5:1–14.
4. Anonymous. Fish Diseases; Emerging viruses. 2013 http://wfrc.usgs.gov/fieldstations/hq/emerging.html (Consulted on Oct 1, 2015).
5. Granzow H, Fichtner D, Schütze H, Lenk M, Dresenkamp B, Nieper H, et al. Isolation and partial characterization of a novel virus from different carp species suffering gill necrosis – ultrastructure and morphogenesis. J Fish Dis. 2014;37:559–69.
6. McCann R Viral survey of fathead minnows, golden shiners, and white suckers from baitfish dealers in Wisconsin. Master's Degree Thesis, 2012 College of Science and Health, University of Wisconsin, La Crosse, USA.
7. Blake S, Bouchard D, Keleher W, Opitz M, Nicholson B. Genomic relationships of the North American isolate of infectious salmon anemia virus (ISAV) to the Norwegian strain of ISAV. Dis Aquat Organ. 1999;35:139–44.
8. Kibenge FSB, Kibenge MJ, McKenna PK, Stothard P, Marshall R, Cusack RR, et al. Antigenic variation among isolates of infectious salmon anaemia virus (ISAV) correlates with genetic variation of the viral haemagglutinin gene. J Gen Virol. 2001;82:2869–79.

9. Kibenge FSB, Godoy MG, Wang Y, Kibenge MJT, Gherardelli V, Mansilla S, et al. Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virol J. 2009;6:88.
10. Kibenge FSB, Kibenge MJT, Wang Y, Qian B, Hariharan S, McGeachy S. Mapping of putative virulence motifs on infectious salmon anaemia virus surface glycoprotein genes. J Gen Virol. 2007;88:3100–11.
11. Nylund A, Plarre H, Karlsen M, Fridell F, Ottem KF, Bratland A, et al. Transmission of infectious salmon anaemia virus (ISAV) in farmed populations of Atlantic salmon (*Salmo salar*). Arch Virol. 2007;152:151–79.
12. Kibenge FSB, Kibenge MJT, Groman D, McGeachy S. *In vivo* correlates of infectious salmon anaemia virus pathogenesis in fish. J Gen Virol. 2006;87:2645–52.
13. Christiansen D, Østergaard PS, Snow MI, Dales OB, Falk K. A low-pathogenic variant of infectious salmon anemia virus (ISAV1 - HPR0) is highly prevalent and causes a non-clinical transient infection in farmed Atlantic salmon (*Salmo salar* L.) in the Faroe Islands. J Gen Virol. 2011;92:909–18.
14. Plarre H, Nylund A, Karlsen M, Brevik Ø, Sæther PA, Vike S. Evolution of infectious salmon anaemia virus (ISA virus). Arch Virol. 2012;157:2309–26.
15. González RR, Ruiz P, Llanos-Rivera A, Cruzat F, Silva J, Astuya A, et al. ISA virus outside the cage: Ichthyofauna and other possible reservoirs to be considered for marine biosafety management in the far-southern ecosystems of Chile. Aquaculture. 2011;318:37–42.
16. Marshall SH, Ramírez R, Labra A, Carmona M, Muñoz C. Bona fide evidence for natural vertical transmission of infectious salmon anemia virus in freshwater brood stocks of farmed Atlantic salmon (Salmo salar) in Southern Chile. J Virol. 2014;88:6012–8.
17. CFIA: ARCHIVED - 2012 – 2013 Status Update on the Surveillance of Wild and Enhanced Anadromous Salmonids in British Columbia. 2014 http://www.inspection.gc.ca/animals/aquatic-animals/diseases/reportable/isa/wild-anadromous-salmonids/eng/1410057185143/1410057186002.
18. Sepúlveda D, Cárdenas C, Carmona M, Marshall SH. Novel strategy to evaluate infectious salmon anemia virus variants by high resolution melting. PLoS One. 2012;7:e37265.
19. Abdelwhab EM, Erfan AM, Grund C, Ziller M, Arafa A-S, Beer M, et al. Simultaneous detection and differentiation by multiplex real time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. Virol J. 2010;7:1–8.
20. Klungthong C, Chinnawirotpisan P, Hussem K, Phonpakobsin T, Manasatienkij W, Ajariyakhajorn C, et al. The impact of primer and probe-template mismatches on the sensitivity of pandemic influenza A/H1N1/2009 virus detection by real-time RT-PCR. J Clin Virol. 2010;48:91–5.
21. Kumar S, Henrickson KJ. Update on influenza diagnostics: lessons from the novel H1N1 influenza A pandemic. Clin Microbiol Rev. 2012;25:344–61.
22. Caraguel C, Stryhn H, Gagné N, Dohoo I, Hammell L. A modeling approach to predict the variation of repeatability and reproducibility of a RT-PCR assay for infectious salmon anaemia virus across infection prevalences and infection stages. Prev Vet Med. 2012;103:63–73.
23. Nérette P, Dohoo I, Hammell L, Gagné N, Barbash P, Maclean S. Estimation of the repeatability and reproducibility of three diagnostic tests for infectious salmon anaemia virus. J Fish Dis. 2005;28:101–10.
24. CFIA: Canada completes infectious salmon anaemia testing: No confirmed cases in BC salmon – December 02, 2011. http://www.inspection.gc.ca/english/corpaffr/newcom/2011/20111202e.shtml.
25. DFO: Accepted Testing Methods for ISAV – November 2011. http://www.dfo-mpo.gc.ca/media/back-fiche/2011/20111108-eng.htm.
26. Kibenge FSB, Munir K, Kibenge MJT, Joseph T, Moneke E. Infectious salmon anaemia virus: causative agent, pathogenesis and immunity. Anim Health Res Rev. 2004;5:65–78.
27. Rimstad E, Mjaaland S. Infectious salmon anaemia virus – an orthomyxovirus causing an emerging infection in Atlantic salmon. Apmis. 2002;110:273–82.
28. Mjaaland S, Hungnes O, Teig A, Dannevig BH, Thorud K, Rimstad E. Polymorphism in the Infectious Salmon Anemia Virus Hemagglutinin Gene: Importance and Possible Implications for Evolution and Ecology of Infectious Salmon Anemia Disease. Virology. 2002;304:379–91.
29. Lyngstad TM, Kristoffersen AB, Hjortaas MJ, Devold M, Aspehaug V, Larssen RB, et al. Low virulent infectious salmon anaemia virus (ISAV-HPR0) is prevalent and geographically structured in Norwegian salmon farming. Dis Aquat Organ. 2012;101:197–206.
30. Gagné N, Ritchie RJ. Development of a strand-specific RT-PCR assay for ISAV. Can Tech Rep Fish Aquat Sci. 2006;2675:1–32.
31. Plarre H, Devold M, Snow M, Nylund A. Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in Western Norway. Dis Aquat Organ. 2005;66:71–9.
32. Leland DS, Ginocchio CC. Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev. 2007;20:49–78.
33. Knüsel R, Bergmann SM, Casey J, Segner H, Wahli T. Virus isolation vs RT-PCR: which method is more successful in detecting VHSV and IHNV in fish tissue sampled under field conditions? J Fish Dis. 2007;30:559–68.
34. Bergh O. The dual myths of the healthy wild fish and the unhealthy farmed fish. Dis Aquat Org. 2007;75:159–64.
35. Garibyan L, Avashia N. Polymerase Chain Reaction. J Invest Dermatol. 2013;133:e6.
36. Snow M, McKay P, McBeath AJ, Black J, Doig F, Kerr R, et al. Development, application and validation of a Taqman® real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (*Salmo salar*). Dev Biol. 2006;126:133–45.
37. Kibenge FSB, Kibenge MJT, Masaoud E. Infectious Salmon Anaemia Virus (ISAV) Ringtest: Validation of the ISAV Diagnostic Process using Virus-spiked Fish Tissues and ISAV TaqMan® Real-time RT-PCR. J Aquac Res and Devel. 2011;2:1–8.
38. OIE: Manual of Diagnostic Tests for Aquatic Animals 2015, http://www.oie.int/international-standard-setting/aquatic-manual/access-online/
39. Devold M, Krossøy B, Aspehaug V, Nylund A. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) I carrier sea trout Salmo trutta after experimental infection. Dis Aquat Org. 2000;40:9–18.
40. Casselman A: More salmon shenanigans in British Columbia. Nature News Blog, December 02, 2011 http://blogs.nature.com/news/2011/12/more_salmon_shenanigans_in_bri.html.
41. Amos KH, Gustafson L, Warg J, Whaley J, Purcell M, Rolland J, et al. U.S. Response to a Report of Infectious Salmon Anemia Virus in Western North America. Fisheries. 2014;39:501–6.
42. Cohen Commission: Exhibits1549, 2040, 2042, 2043, 2044, 2045, 2051, 2052, 2053, 2060 https://web.archive.org/web/20131114212437/http://www.cohencommission.ca/en/Exhibits.php
43. Bates D, Mächler M, Bolker BM, Walker, SC: Fitting Linear Mixed-Effects Models using lme4. https://cran.r-project.org/web/packages/lme4/vignettes/lmer.pdf.
44. EFSA: European Food Safety Authority (EFSA) Panel on Animal Health and Welfare (AHAW): Scientific opinion on infectious salmon anaemia. *EFSA Journal* 2012, 10:2971. http://www.efsa.europa.eu/en/efsajournal/pub/2971.htm.
45. Nylund A, Devold M, Plarre H, Isdal E, Aarseth M. Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. Dis Aquat Org. 2003;56:11–24.
46. Lyngstad TM, Jansen PA, Sindre H, Jonassen CM, Hjortaas MJ, Johnsen S, et al. Epidemiological investigation of infectious salmon anaemia (ISA) outbreaks in Norway 2003–2005. Prev Vet Med. 2008;84:213–27.
47. Rodger HD, Turnbull T, Muir F, Millar S, Richards RH. Infectious salmon anaemia (ISA) in the United Kingdom. B Eur Assoc Fish Pathol. 1998;18:115–6.
48. Cottet L, Cortez-San Martin M, Tello M, Olivares E, Rivas-Aravena A, Vallejos E, et al. Bioinformatic analysis of the genome of infectious salmon anaemia virus associated with outbreaks with high mortality in Chile. J Virol. 2010;84:11916–28.
49. Cunningham CO, Gregory A, Black J, Simpson I, Raynard RS. A novel variant of infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull Eur Ass Fish Pathol. 2002;22:366–74.
50. Castro-Nallar E, Martín MC-S, Mascayano C, Molina C, Crandall KA. Molecular Phylodynamics and protein modeling of infectious salmon anaemia virus (ISAV). BMC Evol Biol. 2011;11:349.
51. Workenhe ST, Kibenge MJT, Iwamoto T, Kibenge FSB. Absolute quantitation of infectious salmon anaemia virus using different real-time reverse transcription PCR chemistries. J Virol Methods. 2008;154:128–34.
52. Kibenge MJT, Iwamoto T, Wang Y, Morton A, Godoy MG, Kibenge FSB. Whole-genome analysis of piscine reovirus (PRV) shows PRV represents a new genus in family *Reoviridae* and its genome segment S1 sequences group it into two separate sub-genotypes. Virol J. 2013;10:230.
53. Kibenge FSB, Dybing JK, McKenna KP. Rapid procedure for large-scale isolation of plasmid DNA. Biotechniques. 1991;11:65–7.

54. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
55. GenBank Database: http://www.ncbi.nlm.nih.gov/Genbank
56. Stothard P. The Sequence Manipulation Suite: JavaScript Programs for Analyzing and Formatting Protein and DNA Sequences. Biotechniques. 2000;28:1102–4. http://www.ualberta.ca/~stothard/javascript/index.html.
57. Pearson WR, Lipman DJ. Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A. 1998;85:2444–8.
58. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res. 1997;25:4876–82.
59. Dannevig BH, Falk K, Namork E. Isolation of the causal virus of infectious salmon anemia (ISA) in a long-term cell line from Atlantic head kidney. J Gen Virol. 1995;76:1353–9.
60. Munir K, Kibenge FSB. Detection of infectious salmon anaemia virus by real-time RT-PCR. J Virol Methods. 2004;117:37–47.
61. Raynard RS, Murray AG, Gregory A. Infectious salmon anemia virus in wild fish from Scotland. Dis Aquat Organ. 2001;46:93–100.
62. Olivier G. Disease interactions between wild and cultured fish - Perspectives from the American Northeast (Atlantic Provinces). Bull Eur Assoc Fish Pathol. 2002;22:103–9.
63. Mullins JE, Groman D, Wadowska D. Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar* L.) in New Brunswick, Canada. Bull Eur Assoc Fish Pathol. 1998;18:110–4.
64. Raynard RS, Murray AG, Gregory A. Infectious salmon anaemia virus in wild fish from Scotland. Dis Aquat Org. 2001;46:93–100.
65. MacLean SA, Brown RW: ISA outbreaks and disease testing of Atlantic salmon in the United States and the West Greenland fishery. Work. Pap. 2002/20. Copenhagen: International Council for the Exploration of the Sea North Atlantic Salmon Working Group. 3 p.
66. MacLean SA, Bouchard DA, Ellis SK. Survey of non-salmonid marine fishes for detection of infectious salmon anaemia virus and other salmonid pathogens. In: Miller O, Cipriano RC, editors. International response to infectious salmon anemia: prevention, control, and eradication: Symposium Proceedings. 3–4 September 2002, New Orleans, LA. Tech Bull. Washington, DC: US Dept Agriculture; 2003. p. 135–44.
67. Bouchard DA, Brockway K, Giray C, Keleher W, Merrill PL. First report of infectious salmon anemia (ISA) in the United States. Bull Eur Assoc Fish Pathol. 2001;21:86–8.
68. Thorud KE, Djupvik HO. Infectious salmon anaemia in Atlantic salmon (Salmo salar L). Bull Eur Assoc Fish Pathol. 1988;8:109–11.
69. Cipriano RC. Antibody against infectious salmon anaemia virus among feral Atlantic salmon (*Salmo salar*). ICES J Mar Sci. 2009;66:865–70.
70. Skall HF: Detection of HPR0 in Denmark and criteria for diagnosis of ISA. In Report on the 15th Annual Meeting of the National Reference Laboratories for Fish Diseases. Copenhagen, Denmark. 2011:48–49.
71. Godoy MG, Aedo A, Kibenge MJT, Groman DB, Yason CV, Grothusen H, et al. First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (*Salmo salar*) in Chile. BMC Vet Res. 2008;4:1–13.
72. Anonymous: ISA hits the Faroes. Fish Farming Int 2000, 47.

eLIFE elifesciences.org

SHORT REPORT

EXHIBIT L-8

Endangered wild salmon infected by newly discovered viruses

Gideon J Mordecai[1]*, Kristina M Miller[2]*, Emiliano Di Cicco[2,3], Angela D Schulze[2], Karia H Kaukinen[2], Tobi J Ming[2], Shaorong Li[2], Amy Tabata[2], Amy Teffer[4], David A Patterson[5], Hugh W Ferguson[6], Curtis A Suttle[1,7,8,9]*

[1]Department of Earth, Ocean and Atmospheric Sciences, University of British Columbia, Vancouver, Canada; [2]Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, Canada; [3]Pacific Salmon Foundation, Vancouver, Canada; [4]Department of Forest Sciences, University of British Columbia, Vancouver, Canada; [5]Fisheries and Oceans Canada, Science Branch, Cooperative Resource Management Institute, School of Resource and Environmental Management, Simon Fraser University, Burnaby, Canada; [6]School of Veterinary Medicine, St. George's University, True Blue, Grenada; [7]Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada; [8]Department of Botany, University of British Columbia, Vancouver, Canada; [9]Institute for the Oceans and Fisheries, University of British Columbia, Vancouver, Canada

Abstract The collapse of iconic, keystone populations of sockeye (*Oncorhynchus nerka*) and Chinook (*Oncorhynchus tshawytscha*) salmon in the Northeast Pacific is of great concern. It is thought that infectious disease may contribute to declines, but little is known about viruses endemic to Pacific salmon. Metatranscriptomic sequencing and surveillance of dead and moribund cultured Chinook salmon revealed a novel arenavirus, reovirus and nidovirus. Sequencing revealed two different arenavirus variants which each infect wild Chinook and sockeye salmon. In situ hybridisation localised arenavirus mostly to blood cells. Population surveys of >6000 wild juvenile Chinook and sockeye salmon showed divergent distributions of viruses, implying different epidemiological processes. The discovery in dead and dying farmed salmon of previously unrecognised viruses that are also widely distributed in wild salmon, emphasizes the potential role that viral disease may play in the population dynamics of wild fish stocks, and the threat that these viruses may pose to aquaculture.
DOI: https://doi.org/10.7554/eLife.47615.001

***For correspondence:** gmordecai@eoas.ubc.ca (GJM); Kristi.Saunders@dfo-mpo.gc.ca (KMM); suttle@science.ubc.ca (CAS)

Competing interests: The authors declare that no competing interests exist.

Funding: See page 12

Received: 11 April 2019
Accepted: 16 July 2019
Published: 03 September 2019

Reviewing editor: Richard A Neher, University of Basel, Switzerland

Introduction

Pacific salmon (*Oncorhynchus* spp.) species have supported coastal ecosystems and Indigenous populations surrounding the North Pacific Ocean for tens of millennia. Today, through their anadromous life history, salmon continue to transport nutrients between aquatic and terrestrial environments (***Cederholm et al., 1999***), supply the primary food sources for orca whales and sea lions (***Wasser et al., 2017***; ***Willson and Halupka, 1995***; ***Chasco et al., 2017***; ***Thomas et al., 2017***) and provide economic livelihoods for local communities (***Noakes et al., 2002***). In the Northeast Pacific, widespread declines of Chinook (*O. tshawytscha*) and sockeye (*O. nerka*) salmon have occurred in the last 30 years, leading some populations to the brink of extirpation (***Peterman and Dorner, 2012***; ***Heard et al., 2007***; ***Miller et al., 2011***; ***Jeffries et al., 2014***), and a cause of great concern to Indigenous groups, commercial and recreational fishers, and the general public. Although the exact number of salmon spawning in rivers is unknown, there are large declines in sockeye salmon over a

eLife digest Keystone species are animals and plants that play a pivotal role in supporting the ecosystems they live in, making their conservation a high priority. Chinook and sockeye salmon are two such species. These fish play a central role in the coastal ecosystems of the Northeast Pacific, where they have supported Indigenous populations for thousands of years.

The last three decades have seen large declines in populations of Chinook and sockeye salmon. One factor that may be involved in these declines is viral infection. In the last ten years, advances in DNA sequencing technologies have led to the discovery of many new viruses, and Mordecai et al. used these technologies to look for new viruses in Pacific salmon.

First, Mordecai et al. looked for viruses in dead and dying salmon from farms and discovered three previously unknown viruses. Next, they screened for these viruses in farmed salmon, hatchery salmon and wild salmon to determine their distribution. Two of the viruses were present in fish from the three sources, while one of the viruses was only found in farmed fish. The fact that the three viruses are distributed differently raises questions about how the viruses are transmitted within and between farmed, hatchery and wild salmon populations.

These findings will aid salmon-conservation efforts by informing the extent to which these viruses are present in wild salmon populations. Future work will focus on determining the risks these viruses pose to salmon health and investigating the potential for exchange between hatchery, farmed and wild salmon populations. While farmed Pacific salmon may pose some transmission risk to their wild counterparts, they also offer the opportunity to study disease processes that are not readily observable in wild salmon. In turn, such data can be used to develop policies to minimize the impact of these infectious agents and improve the survival of wild salmon populations.

DOI: https://doi.org/10.7554/eLife.47615.002

large geographic area (***Peterman and Dorner, 2012***). Similarly, Chinook salmon stocks are at only a small percentage of their historic levels, and more than 50 stocks are extinct (***Heard et al., 2007***).

It is thought that infectious disease may contribute to salmon declines (***Miller et al., 2011***), but little is known about infectious agents, especially viruses, endemic to Pacific salmon. Infectious disease has been identified as a potential factor in poor early marine survival in migratory salmon; an immune response to viruses has been associated with mortality in wild migratory smolts and adults (***Miller et al., 2011***; ***Jeffries et al., 2014***), and in unspecified mortalities of salmon in marine net pens in British Columbia (BC) (***Miller et al., 2017***; ***Di Cicco et al., 2018***). For instance, immune responses to viruses such as Infectious haematopoietic necrosis virus (IHNV) and potentially undiscovered viruses, have been associated with mortality in wild juvenile salmon (***Jeffries et al., 2014***). This is an important observation as mortality of juvenile salmon can be as high as ~90% transitioning from fresh water to the marine environment (***Clark et al., 2016***). Together, these suggest that there are undiscovered viruses which may contribute to decreased survival of Pacific salmon but a concerted effort to look for viruses that may contribute to mortality has been absent.

Here, virus-discovery was implemented to screen for viruses associated with mortality. Together, sequencing of dead or moribund aquaculture salmon and live-sampled wild salmon, in-situ hybridization, and epidemiological surveys revealed that previously unknown viruses, some of which are associated with disease, infect wild salmon from different populations.

Results and discussion

Fish were screened against a viral disease detection biomarker panel (VDD) that elucidates a conserved transcriptional pattern indicative of an immune response to active RNA viral infection (***Miller et al., 2017***). For instance, in a previous study, we showed that 31% of moribund Atlantic salmon were in a viral disease state, and half of these were not known to be positive for any known RNA viruses (***Di Cicco et al., 2018***). Individuals that were strongly VDD-positive, but negative for any known salmon viruses (e.g. Piscine orthoreovirus, Erythrocytic necrosis virus, Infectious pancreatic necrosis virus, Infectious hematopoietic necrosis virus, Infectious salmon anaemia virus and Pacific salmon paramyxovirus) were subject to metatranscriptomic sequencing. The sequencing revealed viral transcripts belonging to members of the *Arenaviridae*, *Nidovirales* and *Reoviridae*,

three evolutionarily divergent groups of RNA viruses (***Figure 1***) that can be highly pathogenic

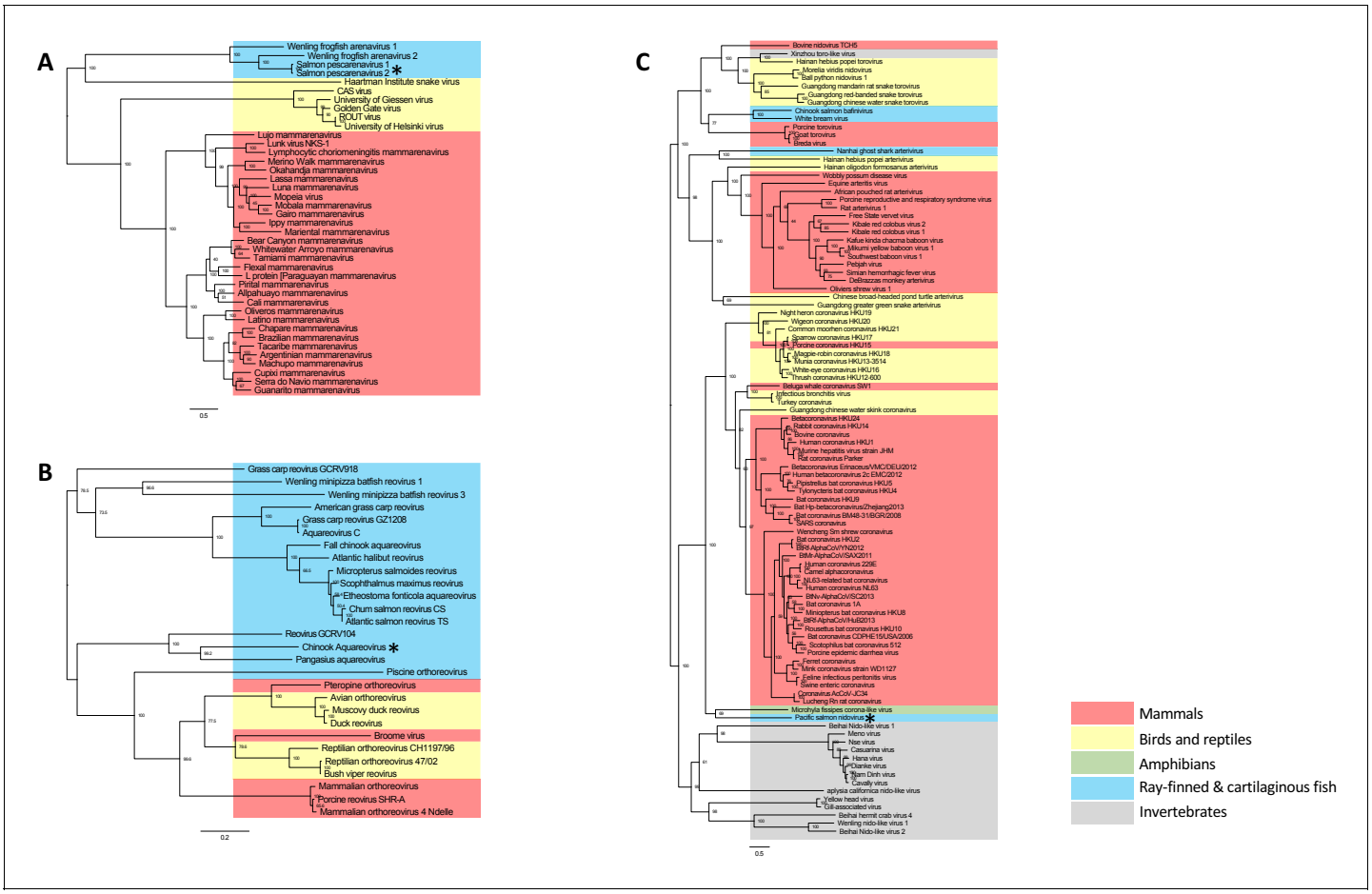


Figure 1. Maximum likelihood phylogenetic tree based on MAFFT alignments of the predicted replicase protein of (**A**) Salmon pescarenavirus and related arenaviruses, (**B**) Chinook Aquareovirus and related Aqua and orthoreoviruses and (**C**) Pacific salmon nidovirus and related Nidovirales. Sequences from this study are marked with an asterisk, scale bar represents the number of amino substitutions per site, node labels show the bootstrap support and host groups are shown by colour. Trees are mid-point rooted, so do not necessarily represent the ancestral relationship of the viruses. Amino acid alignments have been provided in the source data for ***Figure 1***.

DOI: https://doi.org/10.7554/eLife.47615.003

The following source data and figure supplements are available for figure 1:

Source data 1. Arenavirus amino acid alignment.

DOI: https://doi.org/10.7554/eLife.47615.006

Source data 2. Nidovirus amino acid alignment.

DOI: https://doi.org/10.7554/eLife.47615.007

Source data 3. Reovirus amino acid alignment.

DOI: https://doi.org/10.7554/eLife.47615.008

Source data 4. Arenavirus phylogenetic tree.

DOI: https://doi.org/10.7554/eLife.47615.009

Source data 5. Reovirus phylogenetic tree.

DOI: https://doi.org/10.7554/eLife.47615.010

Source data 6. Nidovirus phylogenetic tree.

DOI: https://doi.org/10.7554/eLife.47615.011

Figure supplement 1. Genome organisation and coverage.

DOI: https://doi.org/10.7554/eLife.47615.004

Figure supplement 1—source data 1. Viral genomic nucleotide sequences.

DOI: https://doi.org/10.7554/eLife.47615.005

(*Yun and Walker, 2012*; *Liang et al., 2014*; *Weiss and Leibowitz, 2011*).

One of the challenges of viral discovery in fish is that the proportion of viral transcripts in vertebrate metatranscriptomic libraries is small compared to the number of transcripts from the host and other contaminating sequences (*Geoghegan et al., 2018*; *Zhang et al., 2019*). However, we were able to achieve near-coding complete genomes for the three new viruses (*Figure 1—figure supplement 1A and B*). The genomic organisation of the newly discovered viruses was consistent with related viruses in fish. For instance, SPAV has three genomic segments, as shown for other arenaviruses in fish (*Shi et al., 2018*). High-throughput RT-PCR screening of >6000 wild juvenile Chinook and sockeye salmon showed dissimilar geographical distributions of infected fish, reflecting differences in epidemiological patterns of transmission and infection dynamics for each of the viruses (*Figure 2*).

The distribution and abundance of the different viruses varied markedly. Arenaviruses were relatively common (*Figure 2—figure supplement 1*) and geographically widespread in migratory juvenile Chinook and sockeye salmon in the marine environment (*Figure 2*, *Figure 2—figure supplement 2*). Whereas, the nidovirus was spatially localised and predominantly observed at high prevalence over multiple years in Chinook salmon leaving freshwater hatcheries (*Figure 2*). Finally, the reovirus was detected only in farmed Chinook salmon (*Figure 2* and *Figure 2—figure supplement 1*).

With the exception of their relatively recent discovery in snakes (*Stenglein et al., 2012*) and frogfish (*Shi et al., 2018*), arenaviruses were thought to solely infect mammals. The arenaviruses reported here share less than 15% amino-acid sequence similarity (in the RdRp) to those from mammals and snakes, and define a new monophyletic evolutionary group, the pescarenaviruses (*Figure 1A*). The absence of clear sequence homology in the glycoprotein, the difference in genome segmentation (*Shi et al., 2018*), as well as phylogenetic analysis of the replicase demonstrate that pescarenaviruses share a common but ancient ancestor with arenaviruses infecting snakes and mammals. We recommend these fish-infecting arenaviruses are assigned to the new genus *Pescarenavirus*, with those infecting Chinook and sockeye salmon being assigned to the species *Salmon pescarenavirus* (SPAV), strains 1 and 2, respectively.

Farmed Chinook salmon positive for SPAV-1 displayed pathology and symptoms consistent with disease including inflammation of the spleen and liver, as well as tubule necrosis and hyperplasia in the kidney. Clinically, salmon presented with yellow fluid on the pyloric caeca and swim bladder, pale gills with haemorrhaging on the surface, and anaemia. Wild Chinook and sockeye that tested positive for arenavirus infection, but which were clinically healthy when sampled, showed few histological lesions. In-situ hybridization revealed that arenaviruses were concentrated mainly in macrophage-like cells, melanomacrophages, red-blood cells (RBCs) and endotheliocytes (*Figure 3*). These findings are consistent with localisation of arenaviruses in mammals and snakes, although in contrast to snakes and fish, mammalian red blood cells are not nucleated so the similarity likely only extends to nucleated cells. SPAV-1 and −2 shared similar cell tropism within Chinook and sockeye salmon, respectively (*Figure 3—figure supplement 1*). In one out of the eight Chinook samples examined, moderate chronic-active hepatitis was reported, and staining for SPAV-1 was identified in the area affected by inflammation (*Figure 3C and D*), while in the other samples SPAV-1 was confined to reticuloendothelial cells in the liver tissue or in the sinusoids. More lesions were observed in dead farmed Chinook, where disease progression is more advanced. Our observations indicate that arenaviruses are replicating in red-blood cells, and occur in the macrophages and leukocytes that consume the infected cells. Moreover, the observed pathological changes in arenavirus-infected fish, including anaemia, and lesions in the gills, kidney and liver would be expected for viruses that infect red-blood cells. These results are the first empirical evidence for arenavirus infection in fish, and suggest that SPAV, like many other arenaviruses, has the potential to be a causative agent of disease.

Sequencing of cultured Chinook salmon also revealed a previously undescribed nidovirus and reovirus. Phylogenetic analysis of the reovirus, named *Chinook aquareovirus* (CAV), predicts that it is part of the genus, *Aquareovirus* (*Figure 1B*). Rather than being most closely related to known reoviruses of salmon (*Winton et al., 1981*), CAV groups with a growing number of aquareoviruses, some of which are known to cause haemorrhagic disease and have led to serious losses to aquaculture in China (*Nibert and Duncan, 2013*; *Wang et al., 2012*). The observed clinical signs (anemia, dark spleen, and blood-filled kidneys) in dead farmed Chinook salmon with high loads of CAV are consistent with a haemorrhagic manifestation.

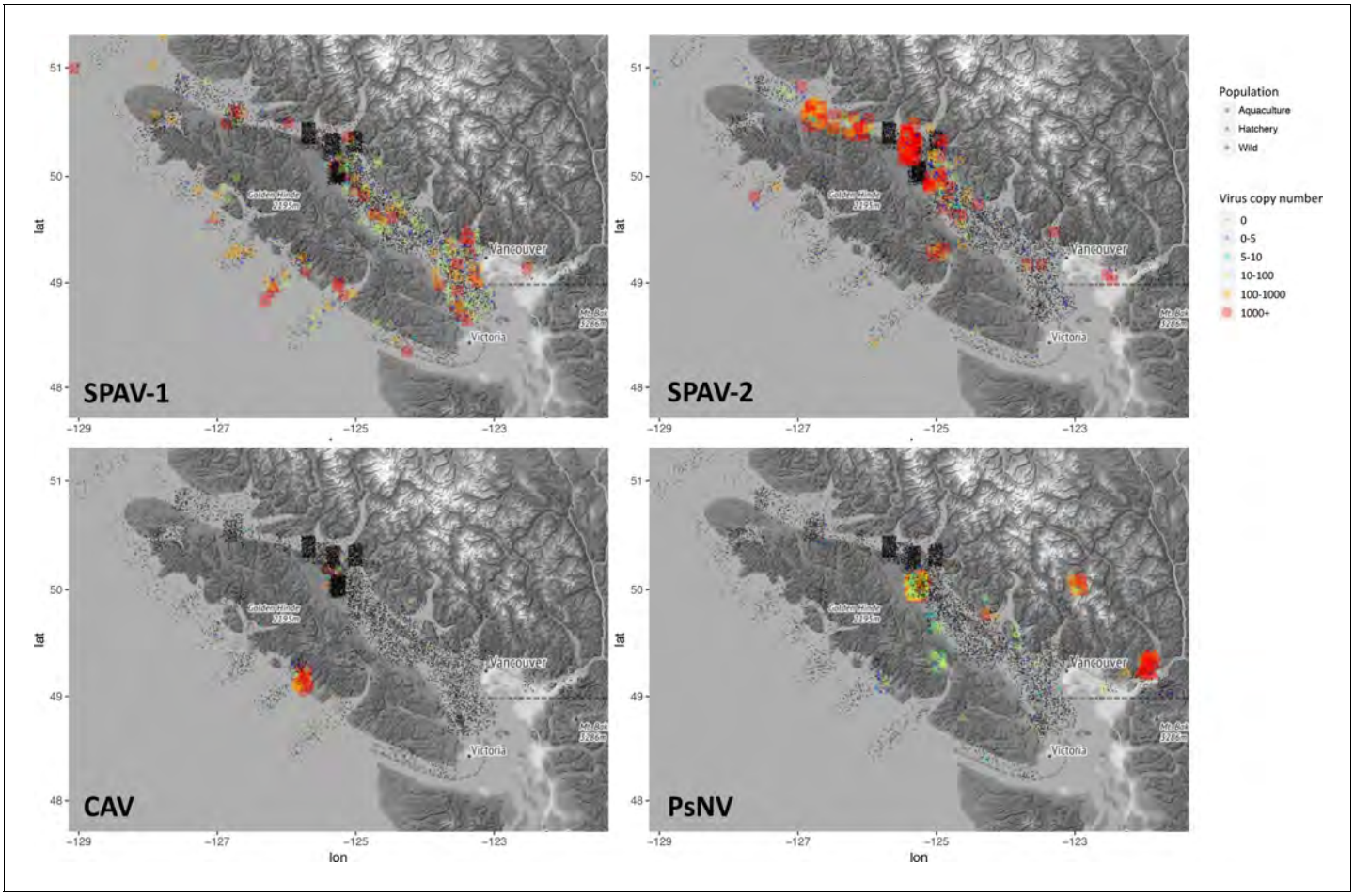


Figure 2. Epidemiological maps of Salmon pescarenavirus 1 and 2 (SPAV-1 and SPAV-2), Chinook aquareovirus (CAV) and Pacific salmon nidovirus (PsNV) around the coast of Vancouver Island. Individual samples are shown at the location collected, negative samples are black, and positive samples are coloured and sized according to the virus copy number. A small degree of random noise was added to the longitude and latitude to prevent overplotting.

DOI: https://doi.org/10.7554/eLife.47615.012

The following source data and figure supplements are available for figure 2:

Source data 1. Source data (RT-PCR copy number and sampling locations) for hte epidemiological maps.

DOI: https://doi.org/10.7554/eLife.47615.015

Figure supplement 1. Summary of RT-PCR for SPAV-1 and −2, PsNV and CAV using the Biomark Fluidigm platform.

DOI: https://doi.org/10.7554/eLife.47615.013

Figure supplement 2. Epidemiological maps from Washington to Alaska of Salmon pescarenavirus 1 and 2 (SPAV-1 and SPAV-2).

DOI: https://doi.org/10.7554/eLife.47615.014

The novel nidovirus, named *Pacific salmon nidovirus* (PsNV), is most closely related to the recently described Microhyla alphaletovirus 1, which forms a sister group to the coronaviruses (***Bukhari et al., 2018***). This sequence, alongside PsNV are basal to all other Nidovirus families, and their long branch length suggests they each belong to a different genus (***Figure 1C***). While not all coronaviruses cause serious disease, many do, such as SARS and MERS, which cause severe respiratory infections (***de Wit et al., 2016***).

Both SPAV-1 and SPAV-2 were relatively widespread along the coast of southwestern British Columbia, in ocean caught Chinook and sockeye salmon. Currently, it is unclear what is driving differences in SPAV-1 and SPAV-2 prevalence among regions, but the virus appears to be transmitted to juvenile salmon throughout southern BC soon after they enter the ocean, a period known to be critical to their survival (***Beamish et al., 2012a***). SPAV-1 was also relatively common in farmed Chinook populations. The distribution of SPAV-1 in wild Chinook populations was more localised to the

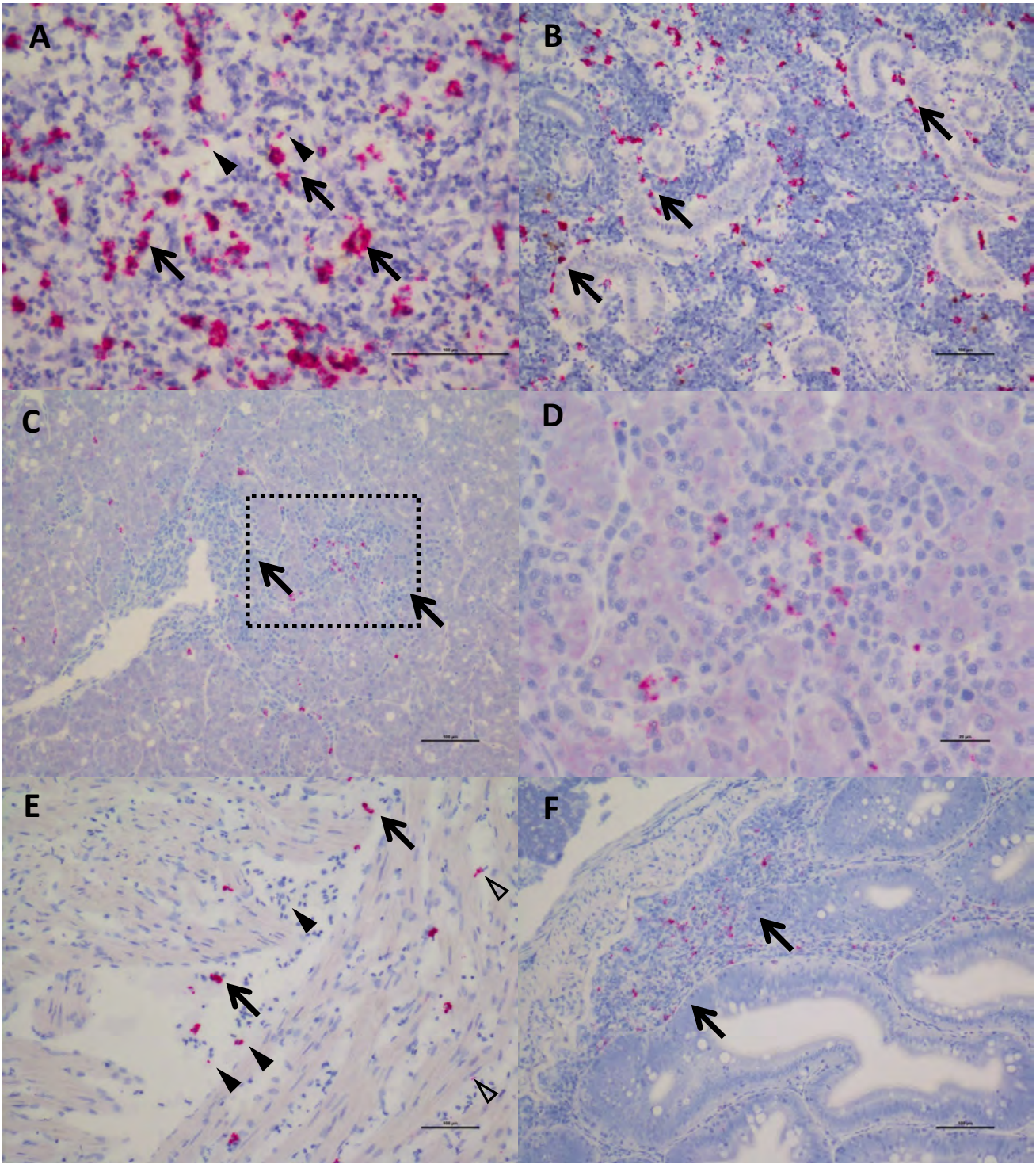


Figure 3. In Situ Hybridization staining of SPAV-1 in Chinook salmon. The red stain indicates localisation of viral RNA as well as viral transcripts. (**A**) Spleen: staining mostly localised in the macrophages (arrows) located around the sinusoids, although scattered positive red blood cells (arrowheads) are also present (scale bar 50 µm). (**B**) Posterior Kidney: the virus appears to be primarily localised in the peritubular capillaries (renal portal vessels) and macrophages (arrows) (scale bar 100 µm). (**C**) Liver: nodules of inflammation are mainly concentrated in a highly marked area. (scale bar 100 µm), dashed rectangle is enlarged in (**D**) showing lymphocytes and macrophages in the inflammatory nodule (several of which are positive for the virus).
Figure 3 continued on next page

Figure 3 continued

(scale bar 20 μm). (**E**) Heart: positive macrophages (arrows) are present between the fibres of the spongy myocardium, along with several positive red blood cells (arrowhead) and endothelial cells (open arrowheads). (scale bar 100 μm). (**F**) Intestine: staining for SPAV-1 is primarily localised to the gut-associated lymphoid tissue (arrows). (scale bar 100 μm).

DOI: https://doi.org/10.7554/eLife.47615.016

The following figure supplement is available for figure 3:

Figure supplement 1. In Situ Hybridization staining of SPAV-2 in sockeye salmon.

DOI: https://doi.org/10.7554/eLife.47615.017

west coast of Vancouver Island than SPAV-2, which was most prevalent on the east coast of Vancouver Island, near the Discovery Islands and the Johnstone Strait, and was rarely detected in sockeye salmon in northern BC and Alaska (***Figure 2—figure supplement 2***).

On the east coast of Vancouver Island, the Johnstone Strait and Discovery Islands have been identified as a potential choke point for the growth and survival of juvenile salmonids (***Healy et al., 2017***). The availability of prey to juvenile sockeye in the northern Johnstone Strait is extremely low, resulting in food limitation and increased competition for prey (***Beamish et al., 2012a***; ***McKinnell et al., 2014***; ***Godwin et al., 2015***; ***Godwin et al., 2018***). These regions of high SPAV-2 infection could represent a stressful part of juvenile sockeye outmigration, possibly resulting in higher susceptibility to infection. Moreover, SPAV-2 was detected at high loads in fish sampled from regions where finfish aquaculture facilities are abundant and accordingly, sea lice infestation is high (***Price et al., 2011***]. It remains an open question whether an alternative host could play a role in virus transmission between fish, and/or result in an increased susceptibility to infection (***Valdes-Donoso et al., 2013***).

The distribution of CAV was markedly different from SPAV. CAV was not detected in any juvenile wild or hatchery Chinook salmon, despite being detected in farmed fish on both the west and east coasts of Vancouver Island. Over 20% of moribund Chinook aquaculture fish tested positive for CAV, with most detections occurring in fish at least 1.5 years after ocean entry, well past the time when migratory salmon were sampled. Hence, infection by CAV may take a considerable time to develop, or be an infection that is only acquired by older fish. CAV was also detected in a small number of farmed Atlantic salmon (seven positive detections of 2816 fish tested). The monophyletic grouping of CAV with other disease causing aquareoviruses and the consistency with haemorrhagic disease suggest that the virus is important to monitor in cultured fish, and potentially wild adults returning after several years at sea.

PsNV distribution was strongly associated with a handful of salmon-enhancement hatcheries but was also detected in 18% of aquaculture Chinook and 3% of wild Chinook (***Figure 2—figure supplement 1***). In hatchery fish, infection by PsNV was primarily localised to gill tissue (***Figure 4A***), reminiscent of the respiratory disease caused by the related mammalian coronaviruses such as MERS and SARS (***Figure 1C***). PsNV is of particular concern as it proliferates while fish are undergoing smoltification, a process during which the gill tissue undergoes cellular reconfiguration to prepare for saltwater. Notably, branchial proliferation of no known cause was noted in some farmed salmon infected with PsNV. In one of the hatcheries, where pre- and post-release sampling took place, the virus increased in prevalence during smolt development in fresh water, was detected shortly post-release, and was barely detected in the month following ocean entry (***Figure 4B***). This suggests that infected fish either cleared the infection, or did not survive after entry into the marine environment. The second interpretation is consistent with the lower rates of ocean survival in fish produced from hatcheries versus wild salmon (***Beamish et al., 2012b***).

Viral disease is a potential threat to wild fish stocks; yet little is known about viruses circulating in wild, farmed, or hatchery salmon. Here, through metatranscriptomic surveys, we reveal several previously unknown viruses that were discovered in dead and dying aquaculture fish, and show them to also occur in wild and hatchery-reared fish. Depending on the viral and host species, the viruses range from being localised to widespread, from infecting <1% to >20% of fish, and being from within the limits of detection to very high loads. Our results are consistent with some of these viruses being causative agents of disease, making it critical to understand their possible roles in salmon mortality and the decline of wild salmon populations, and their potential interactions with net-pen fish farming and hatchery rearing. Viral discovery in moribund individuals followed by extensive

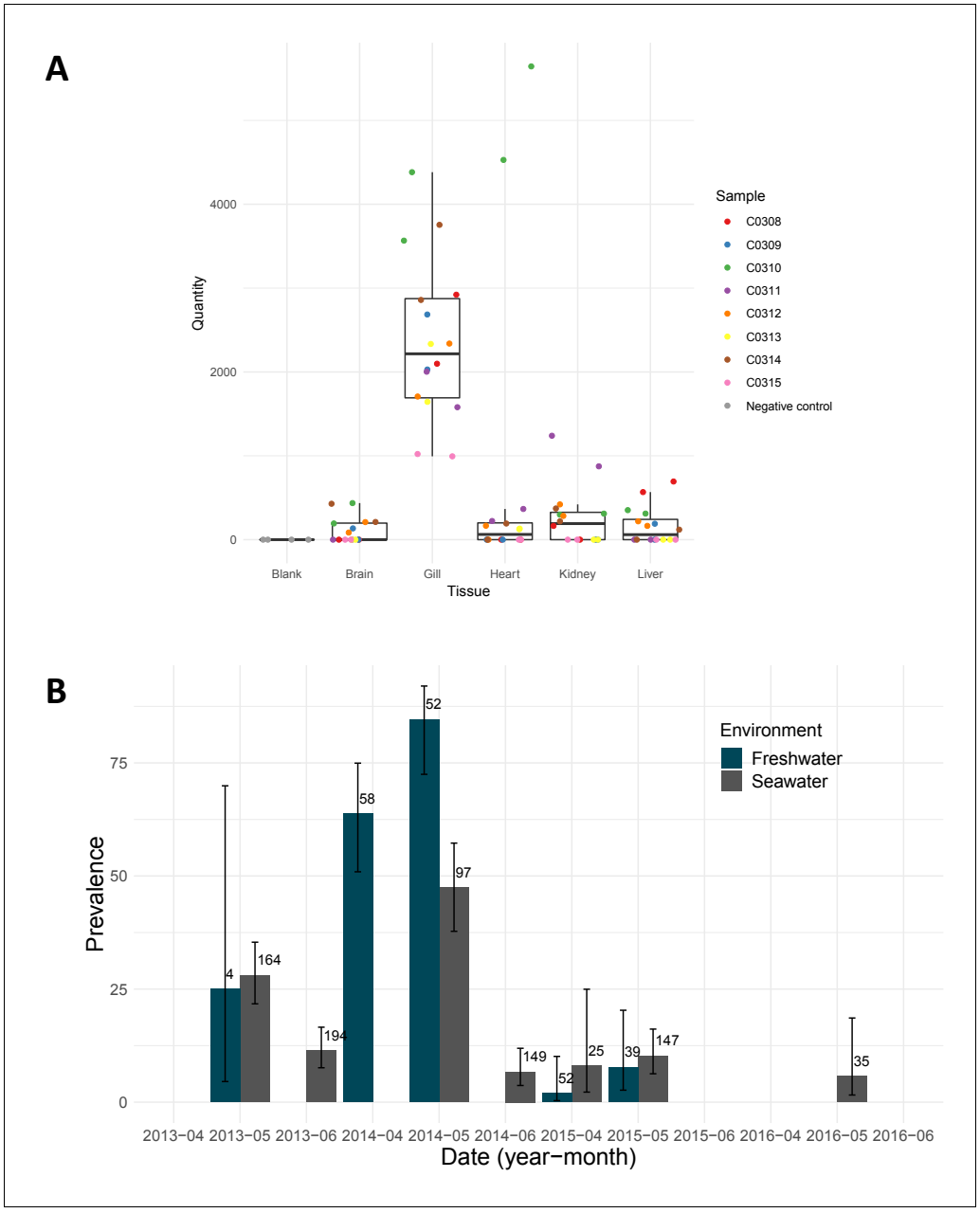


Figure 4. Pacific salmon nidovirus localisation and detections at a single salmon enhancement hatchery. (**A**) Average relative quantity of Pacific salmon nidovirus in dissected tissues of eight Chinook. Each sample was run and plotted in duplicate. (**B**) Prevalence of Pacific salmon nidovirus in fish collected in fresh and saltwater at a single salmon enhancement hatchery over four years. The data shown are the prevalence of positive amplifications above the calculated limit of detection (95%). Numbers show the sample size and error bars show Wilson's binomial confidence intervals.

DOI: https://doi.org/10.7554/eLife.47615.018

surveillance and histopathological localisation are powerful tools towards the ultimate goals of identifying causative agents of disease and understanding the impact of infectious agents in wild populations. These insights are crucial as juvenile salmon that are in less than optimal health are expected to have lower rates of survival in the wild. Continued surveillance and knowledge of endemic and emerging virus infections in these iconic salmon species is beneficial for their conservation.

Materials and methods

Nucleic acid extractions

Samples were provided by the Fisheries and Oceans, Canada Aquaculture Management Division and Salmon Enhancement Program. Additional samples were collected by the Hakai Institute Juvenile Salmon Program. Hatchery samples are identified by fin clipping, and in this study, wild fish could also encompass unmarked hatchery fish. DNA is extracted for detection of DNA viruses, bacteria and parasites from the same tissues from which we extract RNA to target RNA viruses. Nucleic acid extractions on the audit samples (eight tissues-gill, atrium, ventricle, liver, pyloric caeca, spleen, head kidney and posterior kidney) were as previously described (***Laurin et al., 2019***). For the wild Chinook and sockeye samples, homogenization using Tri-reagent was performed in a Mixer Mill (Qiagen, Maryland) on each tissue independently (five tissues- gill, liver, heart, head kidney and brain). Tri-reagent homogenates were organically separated using bromochloropropane, with the RNA-containing aqueous layer removed for RNA extraction and the lower DNA-containing organic layer separated from the organics using a TNES-Urea Buffer (***Asahida et al., 1996***).

For the DNA extractions, a pool of 250 µl (5 tissues contributing 50 µl each) from each of the tissue TNES aqueous layers was processed for DNA using the BioSprint 96 DNA Blood kit (Qiagen, Maryland) and the BioSprint 96 instrument (Qiagen, Maryland) both based on manufacturer's instructions. DNA was quantified using spectrophotometer readings performed on the Infinite M200Pro spectrophotometer (Tecan Group Ltd., Switzerland) and normalised to 62.5 ng/µl using the Freedom Evo (Tecan Group Ltd., Switzerland) liquid handling unit, based on manufacturer's instructions.

Similarly, a pool of 100 µl (5 tissues contributing 20 ul each) of the aqueous layer was processed for RNA using the Magmax−96 for Microarrays RNA kit (Ambion Inc, Austin, TX, USA) with a Biomek NXP (Beckman-Coulter, Mississauga, ON, Canada) automated liquid-handling instrument, both based on manufacturer's instructions. The quantity of RNA was analysed using spectrophotometer readings and normalised to 62.5 ng/µl with a Biomek NXP (Beckman-Coulter, Mississauga, ON, Canada) automated liquid-handling instrument, based on manufacturer's instructions. Mixed tissue RNA (1 µg) was reverse transcribed into cDNA using the superscript VILO master mix kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

Metatranscriptomic sequencing

We applied a panel of host biomarkers (genes) that when co-expressed are indicative of a viral disease state (VDD) (***Miller et al., 2017***). Samples that displayed a positive viral disease state, but were not positive for viruses based on our 45 microbe panel screening, (as described in ***Bass et al., 2019***), were selected for high throughput sequencing of RNA (dual RNA-seq) to discover new viral agents.

Total RNA from the mixed tissue samples was evaluated for quality using the Total RNA Pico chip on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and quantified using the Qubit RNA Br kit (Invitrogen, Carlsbad, CA). A 1/100 dilution of the ERCC RNA Spike-In control mix 1 (Ambion, Carlsbad, CA) was added to each total RNA sample prior to ribosomal depletion and library preparation. The sequencing libraries and ribosomal removal were performed using the Epicentre ScriptSeq Complete Gold Kit (Epidemiology) (Illumina, San Diego, CA) according to manufacturer's instructions and included a positive control (Universal Human Reference RNA) (Agilent, Santa Clara, CA) and negative control (no total RNA). The rRNA depleted total RNA was purified using the Zymo RNA Clean and Concentrate-5 kit (Zymo Research, Irvine, CA) according to manufacturer's instructions and quantified using the Qubit RNA HS kit (Invitrogen, Carlsbad, CA). The ScriptSeq Index reverse primers were added to the cDNA during the final amplification step which involved 14 cycles. The 3'-terminal tagged cDNA and final amplified library were purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA). The final library size was determined using the HS DNA chip on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and the concentration was determined using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA). Sample libraries were normalised to 4 nM, pooled appropriately and denatured and diluted to obtain a final library of 17pM. Prior to loading into a v3 2 × 300 bp kit (Illumina, San Diego, CA), 2% phiX was spiked in. Finally, a paired-end 251 bp sequencing run was performed on the Illumina MiSeq System (Illumina, San Diego, CA), with four samples barcoded and pooled for each run.

To sequence SPAV-2, PsNV and CAV, the samples were prepared using the same method as above but sequenced by BC Cancer Agency using a HiSeq (2 × 125) protocol (four different samples indexed over one lane).

Sequence analysis

The quality of the raw reads was checked using FASTQC (v0.11.7) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low quality reads or regions of adapter sequences were removed using Trimmomatic (v0.36) (**Bankevich et al., 2012**). Reads were aligned to the Atlantic Salmon genome using bwa mem (v0.7.17-r1188) and unmapped reads were retained. The unmapped reads were then balanced using Trimmomatic and assembled into contigs using SPAdes (v3.9.1) genome assembler (**Bankevich et al., 2012**). Putative viral contigs were identified by aligning translated contigs using DIAMOND (v0.9.16.117) (**Buchfink et al., 2015**) to the nr database. Reference alignments of all the reads to the viral contigs were used to ensure that no assembly artefacts occurred and the contigs were trimmed appropriately using Geneious (V10.1.3). Assembled sequences are available on Genbank (BioProject: PRJNA547678, Genbank accession numbers: MK611979 - MK611996) and raw sequencing reads have been uploaded to the Sequence Read Archive (SAMN11974798 - SAMN11974801).

Phylogenetic analysis

The phylogeny of each virus was resolved based on the predicted replicase (CAV and SPAV) and ORF1ab (PsNV) amino acid sequences, as nucleotide sequences were too dissimilar to reliably align. Alignments were generated with MAFTT (v7.42) (**Katoh and Standley, 2013**) employing the E-INS-i algorithm. This alignment algorithm is suited for evolutionarily distinct sequences with conserved motifs (such as viral RNA polymerase) that are embedded within long unalignable residues. The novel salmon viruses were aligned with other viral genomes with shared amino acid similarity as detected by DIAMOND (**Buchfink et al., 2015**). In addition, viral genomes which are known to be evolutionarily related to these were included. The multiple protein alignments were then used as to build phylogenies using PhyML 3.0 (**Guindon et al., 2010**) plugin within Geneious with 100 bootstraps to generate branch support values. Trees are mid-point rooted for clarity only, and do not necessarily represent the ancestral relationship of the viruses.

Assay development and screening

Assembled viral sequence contigs from the appropriate sample were imported into Primer Express v3.0.1 software (Thermo Fisher Scientific, Waltham, MA) where qPCR Taqman assays were designed using default parameters (**Supplementary file 1**). These assays were then tested using the Fluidigm BioMark microfluidics-based qPCR system following the same protocol as described below except with the new viral primer pairs included in the STA step and controls. From these initial screens, the most consistent assay was chosen and APC standards were constructed to include in future Fluidigm BioMark qPCR microbe panels. The assay-specific theoretical limit of detection was calculated as previously described (**Miller et al., 2016**). The limit of detection was applied to categorise fish with amplifications above the 95% detection threshold that is the concentration of the analyte in the sample matrix that would be detected with high statistical certainty (95% of the time). Epidemiological maps were generated using these data with the limit of detection applied. The maps were created within R using ggplot2 (**Wickham, 2016**) and ggmap (**Kahle and Wickham, 2013**).

RT-PCR

For all samples, after reverse transcription, resultant cDNA was combined with the normalised DNA in a ratio of 1:1 and used as the template for the specific target amplification (STA) step. The STA involves a pre-amplification of all primers to be run on a single dynamic array at low concentrations (0.2 µM of each of the primers), and upon completion, excess primers were removed by treating with Exo-SAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions and then diluted 1:5 in DNA re- suspension buffer (Teknova, Hollister, CA).

The 96.96 gene expression dynamic array (Fluidigm Corporation, CA, US) was run according to the procedure outlined previously (**Miller et al., 2016**). Specifically, a 5 µl template mixture was

prepared for each sample containing 1 × TaqMan Universal Master Mix (No UNG), 1 × GE Sample Loading Reagent (Fluidigm PN 85000746) and each of diluted STA'd sample mixtures. Five µl of Assay mix was prepared with 1 × each of the appropriate TaqMan qPCR assays (agent probe in FAM-MGB and artificial positive construct (APC) probe in NED-MGB, 10 µM of primers and 3 µM of probes) and 1 × Assay Loading Reagent (Fluidigm PN 85000736).

Controls were added prior to running the dynamic array (***Miller et al., 2016***). Note, APC clones to all assays were contained in a single serially diluted pool, loaded last, minimising the likelihood of contamination of any single APC clone. Once loading and mixing of the dynamic array was completed within the IFC HX controller, the array was transferred to the BioMark HD instrument and processed using the GE 96 × 96 Standard TaqMan program for qPCR which includes a hot start followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min (Fluidigm Corporation, CA, USA). The data were analysed with Real-Time PCR Analysis Software (Fluidigm Corporation, CA, USA).

Chinook smolt samples positive for PsNV from 2014 were used for tissue localization (***Figure 4A***). Gill, liver, heart, kidney, and brain were individually homogenized, processed for RNA extraction (as described above), and 1 ug normalised RNA was used for reverse transcription. Resultant cDNA for each individual tissue was used as the template for PsNV relative quantification using an ABI 7900HT (ABI) in 384-well optical plates. The qPCR reaction volume was 12 µl, which comprised 6 µl of 2X TaqMan Gene Expression Master Mix (ABI PN 4369016), 4.3 µl of water, 0.22 µl of mixed forward and reverse primers (900 nM final concentration of each), 0.24 µl of each probe (200 nM final concentration; assay specific probe and APC control probe), and 1 µl of cDNA template. Temperature cycles included one 2 min hold (50˚C), a 10 min denaturation (95˚C), and 40 cycles of denaturation (95˚C for 15 s), annealing and extension (60˚C for 60 s). Amplification conditions on the ABI 7900 were not optimised for this platform, but rather closely reflected those used on the BioMark platform. Samples run on the ABI did not undergo STA enrichment. Standard curves were constructed using the same APC clone standards spiked in with CHSE DNA as on the BioMark. Serial dilutions were made to obtain concentrations of 24, 1.2 × 102, 6 × 102, 3 × 103, 1.5 × 104, 1.5 × 105 copies of the clone per reaction. Clone standards, unknown samples, positive and negative controls were all run in duplicate. The ABI software calculates the relative copy number based upon the serial dilution of the standard curve.

Histopathology

Before the discovery of these viruses clinical signs of disease and histopathological lesions were assessed for approximately 230 farmed Chinook salmon sampled in the Audit program. Consequently, gills, skeletal muscle, spleen, liver, heart, anterior and posterior kidney, pyloric caeca and brain from eleven samples of Chinook (eight wild fish and three farmed fish) and ten sockeye (all wild fish) positive for SPAV were histopathologically analysed to assess the presence of lesions. All tissues were fixed in 10% neutral buffered formalin, dehydrated through an ascending gradient of alcohol solutions, embedded in paraffin wax, cut at 3.5 µm thickness, and stained with routine hematoxylin and eosin (H and E) for morphological evaluation by light microscope.

In Situ Hybridization (ISH)

RNA-ISH was performed using RNAscope 2.5 HD Duplex assay (Advanced Cell Diagnostics, Newark, California, USA, catalog# 322500) according to the manufacturer's instructions. Briefly, consecutive sections of Chinook and sockeye salmon samples utilised for the histopathological analysis were dewaxed by incubating for 60 min at 60˚C and endogenous peroxidases were quenched with hydrogen peroxide for 10 min at room temperature. Slides were then boiled for 30 min in RNAscope target retrieval reagents (Advanced Cell Diagnostics, Newark, California, USA) and incubated for 30 min in RNAscope Protease Plus reagent prior to hybridization. The slides underwent hybridization with RNAscope probes against a portion of SPAV-1 and SPAV-2 genome (Advanced Cell Diagnostics, Newark, California, USA, catalog #513591-C2 and 538881-C2, respectively). A RNAscope probe against Coil-p84 housekeeping gene in Chinook salmon (Advanced Cell Diagnostics, Newark, California, USA, catalog #512391) was used as positive control probe to confirm the efficacy of the probes and the viability of the samples. Two samples which were negative for SPAV-1 and SPAV-2 were used as negative controls to confirm absence of background and (or) non-specific cross-

reactivity of the assay. Signal amplification was performed according to the manufacturer's instructions, followed by counterstaining with Gill's hematoxylin and visualisation by bright field microscopy.

Acknowledgements

We thank Jeffrey Joy, Veronica Paglowski, Jan Finke, Thomas Waltzek, Ben Neuman and Humberto Debat for advice and help with the analysis, DFO Aquaculture Management Division, Salmon Enhancement Program and the Hakai Institute Juvenile Salmon Program for provision of samples, and all of the vessel and field crews who collected the thousands of samples. We thank Amy Chan and all members of the Suttle lab for their feedback as the project developed. This research was supported by funding for the Strategic Salmon Health Initiative (SSHI), which is part of the Salish Sea Marine Survival Project. The SSHI seeks to discover microbes present in salmon in British Columbia that may be undermining the productivity of Pacific salmon, and is funded by the Pacific Salmon Foundation (PSF), Genome British Columbia, and Fisheries and Oceans, Canada. This is publication number 32 from the Salish Sea Marine Survival Project (marinesurvivalproject.com).

Additional information

Funding

Funder	Grant reference number	Author
Pacific Salmon Foundation	SSHI	Gideon J Mordecai Kristina M Miller Emiliano DiCicco Angela D Schulze Karia H Kaukinen Tobi J Ming Shaorong Li Amy Tabata Amy Teffer
Genome British Columbia	SSHI	Gideon J Mordecai Kristina M Miller Emiliano DiCicco Angela D Schulze Karia H Kaukinen Tobi J Ming Shaorong Li Amy Tabata Amy Teffer
Mitacs	SSHI	Gideon J Mordecai

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Gideon J Mordecai, Conceptualization, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing, Led the analyses and prepared the figures; Kristina M Miller, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Project administration, Writing—review and editing, Conceived the study; Emiliano Di Cicco, Formal analysis, Investigation, Writing—original draft, Conducted pathology, histopathology and in situ hybridisation; Angela D Schulze, Investigation, Methodology, Performed sequencing, nucleic acid extractions and molecular analyses; Karia H Kaukinen, Investigation, Methodology, Conducted field sampling, nucleic acid extractions and molecular analyses including tissue localisation by RT-PCR; Tobi J Ming, Investigation, Methodology, Conducted field sampling, nucleic acid extractions and molecular analyses; Shaorong Li, Data curation, Investigation, Methodology, Conducted nucleic acid extractions and molecular analyses; Amy Tabata, Data curation, Conducted field sampling, nucleic acid extractions and molecular analyses; Amy Teffer, Investigation, Writing—review and editing, Conducted field sampling, nucleic acid extractions and molecular analyses; David A Patterson,

Conceptualization, Investigation, Writing—review and editing, Conducted field sampling, nucleic acid extractions and molecular analyses; Hugh W Ferguson, Investigation, Writing—review and editing, Conducted pathology, histopathology and in situ hybridisation; Curtis A Suttle, Resources, Supervision, Writing—review and editing, Conceived the study

Author ORCIDs
Gideon J Mordecai https://orcid.org/0000-0001-8397-9194
Curtis A Suttle https://orcid.org/0000-0002-0372-0033

Decision letter and Author response
Decision letter https://doi.org/10.7554/eLife.47615.061
Author response https://doi.org/10.7554/eLife.47615.062

Additional files

Supplementary files

- Supplementary file 1. Table of primers and taqman assays used in this study.
DOI: https://doi.org/10.7554/eLife.47615.019
- Supplementary file 2. Commands used in the bioinformatic pipeline.
DOI: https://doi.org/10.7554/eLife.47615.020
- Transparent reporting form
DOI: https://doi.org/10.7554/eLife.47615.021

Data availability

Assembled viral genomes have been deposited to Genbank under accession numbers MK611979–MK611996 and sequencing reads have been submitted to the Sequence Read Archive under the accession: PRJNA547678.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/sra/PRJNA547678	NCBI Sequence Read Archive, PRJNA547678
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611979	NCBI Genbank, MK611979
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson,	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611980	NCBI Genbank, MK611980

Hugh W Ferguson, Curtis A Suttle				
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611981	NCBI Genbank, MK611981
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611982	NCBI Genbank, MK611982
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611983	NCBI Genbank, MK611983
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611984	NCBI Genbank, MK611984
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611985	NCBI Genbank, MK611985
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611986	NCBI Genbank, MK611986
Gideon J Mordecai, Kristina M Miller,	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=	NCBI Genbank, MK611987

Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle			MK611987	
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611988	NCBI Genbank, MK611988
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611989	NCBI Genbank, MK611989
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611990	NCBI Genbank, MK611990
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611991	NCBI Genbank, MK611991
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611992	NCBI Genbank, MK611992
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611993	NCBI Genbank, MK611993

Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle				
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611994	NCBI Genbank, MK611994
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611995	NCBI Genbank, MK611995
Gideon J Mordecai, Kristina M Miller, Emiliano Di Cicco, Angela D Schulze, Karia H Kaukinen, Tobi J Ming, Shaorong Li, Amy Tabata, Amy Teffer, David A Patterson, Hugh W Ferguson, Curtis A Suttle	2019	Endangered wild salmon infected by newly discovered viruses	https://www.ncbi.nlm.nih.gov/nuccore/?term=MK611996	NCBI Genbank, MK611996

References

Asahida T, Kobayashi T, Saitoh K, Nakayama I. 1996. Tissue preservation and total DNA extraction form fish stored at ambient temperature using buffers containing high concentration of urea. *Fisheries Science* **62**:727–730. DOI: https://doi.org/10.2331/fishsci.62.727

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin AV, Sirotkin AV, Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology* **19**:455–477. DOI: https://doi.org/10.1089/cmb.2012.0021, PMID: 22506599

Bass AL, Hinch SG, Teffer AK, Patterson DA, Miller KM. 2019. Fisheries capture and infectious agents are associated with travel rate and survival of chinook salmon during spawning migration. *Fisheries Research* **209**: 156–166. DOI: https://doi.org/10.1016/j.fishres.2018.09.009

Beamish RJ, Neville C, Sweeting R, Lange K. 2012a. The synchronous failure of juvenile Pacific salmon and herring production in the strait of Georgia in 2007 and the poor return of sockeye salmon to the fraser river in 2009. *Marine and Coastal Fisheries* **4**:403–414. DOI: https://doi.org/10.1080/19425120.2012.676607

Beamish RJ, Sweeting RM, Neville CM, Lange KL, Beacham TD, Preikshot D. 2012b. Wild chinook salmon survive better than hatchery salmon in a period of poor production. *Environmental Biology of Fishes* **94**:135–148. DOI: https://doi.org/10.1007/s10641-011-9783-5

Buchfink B, Xie C, Huson DH. 2015. Fast and sensitive protein alignment using DIAMOND. *Nature Methods* **12**: 59–60. DOI: https://doi.org/10.1038/nmeth.3176, PMID: 25402007

Bukhari K, Mulley G, Gulyaeva AA, Zhao L, Shu G, Jiang J, Neuman BW. 2018. Description and initial characterization of metatranscriptomic nidovirus-like genomes from the proposed new family Abyssoviridae, and from a sister group to the Coronavirinae, the proposed genus Alphaletovirus. *Virology* **524**:160–171. DOI: https://doi.org/10.1016/j.virol.2018.08.010, PMID: 30199753

Cederholm CJ, Kunze MD, Murota T, Sibatani A. 1999. Pacific Salmon carcasses: essential contributions of nutrients and energy for aquatic and terrestrial ecosystems. *Fisheries* **24**:6–15. DOI: https://doi.org/10.1577/1548-8446(1999)024<0006:PSC>2.0.CO;2

Chasco BE, Kaplan IC, Thomas AC, Acevedo-Gutiérrez A, Noren DP, Ford MJ, Hanson MB, Scordino JJ, Jeffries SJ, Marshall KN, Shelton AO, Matkin C, Burke BJ, Ward EJ. 2017. Competing tradeoffs between increasing

marine mammal predation and fisheries harvest of chinook salmon. *Scientific Reports* **7**:15439. DOI: https://doi.org/10.1038/s41598-017-14984-8, PMID: 29158502

Clark TD, Furey NB, Rechisky EL, Gale MK, Jeffries KM, Porter AD, Casselman MT, Lotto AG, Patterson DA, Cooke SJ, Farrell AP, Welch DW, Hinch SG. 2016. Tracking wild sockeye salmon smolts to the ocean reveals distinct regions of nocturnal movement and high mortality. *Ecological Applications* **26**:959–978. DOI: https://doi.org/10.1890/15-0632, PMID: 27509741

de Wit E, van Doremalen N, Falzarano D, Munster VJ. 2016. SARS and MERS: recent insights into emerging coronaviruses. *Nature Reviews Microbiology* **14**:523–534. DOI: https://doi.org/10.1038/nrmicro.2016.81, PMID: 27344959

Di Cicco E, Ferguson HW, Kaukinen KH, Schulze AD, Li S, Tabata A, Günther OP, Mordecai G, Suttle CA, Miller KM. 2018. The same strain of *Piscine orthoreovirus* (PRV-1) is involved in the development of different, but related, diseases in Atlantic and Pacific Salmon in British Columbia . *Facets* **3**:599–641. DOI: https://doi.org/10.1139/facets-2018-0008

Geoghegan JL, Di Giallonardo F, Cousins K, Shi M, Williamson JE, Holmes EC. 2018. Hidden diversity and evolution of viruses in market fish. *Virus Evolution* **4**:vey031. DOI: https://doi.org/10.1093/ve/vey031, PMID: 30397510

Godwin SC, Dill LM, Reynolds JD, Krkošek M. 2015. Sea lice, Sockeye Salmon, and foraging competition: lousy fish are lousy competitors. *Canadian Journal of Fisheries and Aquatic Sciences* **72**:1113–1120. DOI: https://doi.org/10.1139/cjfas-2014-0284

Godwin SC, Krkošek M, Reynolds JD, Rogers LA, Dill LM. 2018. Heavy sea louse infection is associated with decreased stomach fullness in wild juvenile sockeye salmon. *Canadian Journal of Fisheries and Aquatic Sciences* **75**:1587–1595. DOI: https://doi.org/10.1139/cjfas-2017-0267

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**:307–321. DOI: https://doi.org/10.1093/sysbio/syq010, PMID: 20525638

Healy SJ, Hinch SG, Porter AD, Rechisky EL, Welch DW, Eliason EJ, Lotto AG, Furey NB. 2017. Route-specific movements and survival during early marine migration of hatchery steelhead Oncorhynchus mykiss smolts in coastal british columbia. *Marine Ecology Progress Series* **577**:131–147. DOI: https://doi.org/10.3354/meps12238

Heard WR, Shevlyakov E, Zikunova OV, McNicol RE. 2007. Chinook salmon-trends in abundance and biological characteristics. *North Pacific Anadromous Fish Commission Bulletin* **4**:77–91.

Jeffries KM, Hinch SG, Gale MK, Clark TD, Lotto AG, Casselman MT, Li S, Rechisky EL, Porter AD, Welch DW, Miller KM. 2014. Immune response genes and pathogen presence predict migration survival in wild salmon smolts. *Molecular Ecology* **23**:5803–5815. DOI: https://doi.org/10.1111/mec.12980, PMID: 25354752

Kahle D, Wickham H. 2013. Ggmap: spatial visualization with ggplot2. *The R Journal* **5**:144. DOI: https://doi.org/10.32614/RJ-2013-014

Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**:772–780. DOI: https://doi.org/10.1093/molbev/mst010, PMID: 23329690

Laurin E, Jaramillo D, Vanderstichel R, Ferguson H, Kaukinen KH, Schulze AD, Keith IR, Gardner IA, Miller KM. 2019. Histopathological and novel high-throughput molecular monitoring data from farmed salmon (Salmo salar and Oncorhynchus spp.) in British Columbia, Canada, from 2011–2013. *Aquaculture* **499**:220–234. DOI: https://doi.org/10.1016/j.aquaculture.2018.08.072

Liang HR, Li YG, Zeng WW, Wang YY, Wang Q, Wu SQ. 2014. Pathogenicity and tissue distribution of grass carp reovirus after intraperitoneal administration. *Virology Journal* **11**:178. DOI: https://doi.org/10.1186/1743-422X-11-178, PMID: 25293723

McKinnell S, Curchitser E, Groot K, Kaeriyama M, Trudel M. 2014. Oceanic and atmospheric extremes motivate a new hypothesis for variable marine survival of fraser river sockeye salmon. *Fisheries Oceanography* **23**:322–341. DOI: https://doi.org/10.1111/fog.12063

Miller KM, Li S, Kaukinen KH, Ginther N, Hammill E, Curtis JM, Patterson DA, Sierocinski T, Donnison L, Pavlidis P, Hinch SG, Hruska KA, Cooke SJ, English KK, Farrell AP. 2011. Genomic signatures predict migration and spawning failure in wild canadian salmon. *Science* **331**:214–217. DOI: https://doi.org/10.1126/science.1196901, PMID: 21233388

Miller KM, Gardner IA, Vanderstichel R, Burnley TA, Ginther NG. 2016. *Report on the Performance Evaluation of the Fluidigm BioMark Platform for High-Throughput Microbe Monitoring in Salmon*: Technical Report.

Miller KM, Günther OP, Li S, Kaukinen KH, Ming TJ. 2017. Molecular indices of viral disease development in wild migrating salmon†. *Conservation Physiology* **5**:cox036. DOI: https://doi.org/10.1093/conphys/cox036, PMID: 28702195

Nibert ML, Duncan R. 2013. Bioinformatics of recent aqua- and Orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications. *PLOS ONE* **8**:e68607. DOI: https://doi.org/10.1371/journal.pone.0068607, PMID: 23861926

Noakes DJ, Beamish RJ, Gregory R. 2002. *British Columbia's commercial salmon industry*: North Pacific Anadromous Fish Commission. DOI: https://doi.org/10.13140/RG.2.2.16087.47526

Peterman RM, Dorner B. 2012. A widespread decrease in productivity of sockeye salmon (*Oncorhynchus nerka*) populations in western North America . *Canadian Journal of Fisheries and Aquatic Sciences* **69**:1255–1260. DOI: https://doi.org/10.1139/f2012-063

Price MH, Proboszcz SL, Routledge RD, Gottesfeld AS, Orr C, Reynolds JD. 2011. Sea louse infection of juvenile sockeye salmon in relation to marine salmon farms on Canada's west coast. *PLOS ONE* **6**:e16851. DOI: https://doi.org/10.1371/journal.pone.0016851, PMID: 21347456

Shi M, Lin XD, Chen X, Tian JH, Chen LJ, Li K, Wang W, Eden JS, Shen JJ, Liu L, Holmes EC, Zhang YZ. 2018. The evolutionary history of vertebrate RNA viruses. *Nature* **556**:197–202. DOI: https://doi.org/10.1038/s41586-018-0012-7, PMID: 29618816

Stenglein MD, Sanders C, Kistler AL, Ruby JG, Franco JY, Reavill DR, Dunker F, Derisi JL. 2012.In Identification, characterization, and in vitro culture of highly divergent arenaviruses from Boa constrictors and annulated tree boas: candidate etiological agents for snake inclusion body disease. *mBio* **3**:e00180. DOI: https://doi.org/10.1128/mBio.00180-12, PMID: 22893382

Thomas AC, Nelson BW, Lance MM, Deagle BE, Trites AW. 2017. Harbour seals target juvenile salmon of conservation concern. *Canadian Journal of Fisheries and Aquatic Sciences* **74**:907–921. DOI: https://doi.org/10.1139/cjfas-2015-0558

Valdes-Donoso P, Mardones FO, Jarpa M, Ulloa M, Carpenter TE, Perez AM. 2013. Co-infection patterns of infectious Salmon anaemia and sea lice in farmed Atlantic Salmon, Salmo salar L., in southern Chile (2007-2009). *Journal of Fish Diseases* **36**:353–360. DOI: https://doi.org/10.1111/jfd.12070, PMID: 23347268

Wang Q, Zeng W, Liu C, Zhang C, Wang Y, Shi C, Wu S. 2012. Complete genome sequence of a reovirus isolated from grass carp, indicating different genotypes of GCRV in China. *Journal of Virology* **86**:12466. DOI: https://doi.org/10.1128/JVI.02333-12, PMID: 23087123

Wasser SK, Lundin JI, Ayres K, Seely E, Giles D, Balcomb K, Hempelmann J, Parsons K, Booth R. 2017. Population growth is limited by nutritional impacts on pregnancy success in endangered southern resident killer whales (Orcinus orca). *PLOS ONE* **12**:e0179824. DOI: https://doi.org/10.1371/journal.pone.0179824, PMID: 28662095

Weiss SR, Leibowitz JL. 2011. Coronavirus pathogenesis. *Advances in Virus Research* **81**:85–164. DOI: https://doi.org/10.1016/B978-0-12-385885-6.00009-2, PMID: 22094080

Wickham H. 2016. *Ggplot2: Elegant Graphics for Data Analysis*. Second Edition. Springer.

Willson MF, Halupka KC. 1995. Anadromous fish as keystone species in vertebrate communities. *Conservation Biology* **9**:489–497. DOI: https://doi.org/10.1046/j.1523-1739.1995.09030489.x

Winton JR, Lannan CN, Fryer JL, Kimura T. 1981. Isolation of a new reovirus from chum salmon in Japan. *Fish Pathology* **15**:155–162. DOI: https://doi.org/10.3147/jsfp.15.155

Yun NE, Walker DH. 2012. Pathogenesis of lassa fever. *Viruses* **4**:2031–2048. DOI: https://doi.org/10.3390/v4102031, PMID: 23202452

Zhang YZ, Chen YM, Wang W, Qin XC, Holmes EC. 2019. Expanding the RNA virosphere by unbiased metagenomics. *Annual Review of Virology*. DOI: https://doi.org/10.1146/annurev-virology-092818-015851, PMID: 31100994