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Inactivation of Infectious Hematopoietic Necrosis Virus by Low Levels of Iodine

WILLIAM N. BATTS,[1]* MARSHA L. LANDOLT,[2] AND JAMES R. WINTON[1]

National Fisheries Research Center, Building 204, Naval Station, U.S. Fish and Wildlife Service, Seattle, Washington 98115,[1] and School of Fisheries, University of Washington, Seattle, Washington 98195[2]

Received 17 December 1990/Accepted 12 February 1991

The fish rhabdovirus infectious hematopoietic necrosis virus (IHNV) was rapidly inactivated by extremely low concentrations of iodine in water. A 99.9% virus reduction was obtained in 7.5 s when virus (10^5 PFU/ml) and iodine (0.1 mg/liter, final concentration) were combined in distilled-deionized or hatchery water. Iodine efficacy decreased at pHs greater than 7.5 or when proteinaceous material was added to the water. Bovine serum albumin blocked iodine inactivation of the virus more effectively than did equal concentrations of fetal bovine serum or river sediment. Sodium thiosulfate effectively neutralized free iodine. Powder, iodophor, and crystalline iodine solutions inactivated IHNV equally. Iodine rapidly inactivated IHNV isolates representing each of the five electropherotypes. Under the conditions used in this study, inactivation was not affected by temperature, salinity, or water hardness. When Dworshak National Fish Hatchery water was continuously treated to provide a free iodine concentration of 0.14 mg/liter, a 7.5-s exposure to iodine was sufficient to inactivate 99.9% of the IHNV. Iodine added to water that contained IHNV prevented infection of rainbow trout (*Oncorhynchus mykiss*) fry. These results suggest that the waterborne route of IHNV transmission can be blocked by adding low iodine concentrations to the water supplies of hatcheries.

Millions of fish die each year from the salmonid disease infectious hematopoietic necrosis (IHN). These deaths result directly from mortality and indirectly from mandated destruction of infected fish. The causative agent, infectious hematopoietic necrosis virus (IHNV), is a rhabdovirus that is enzootic in many watersheds of western North America (25, 33).

To control IHN, it is important to understand the epizootiology of the virus. Two modes of transmission are known. The first is vertical transmission, in which the virus is passed from adult to offspring by gametes (20). Surface disinfection of fertilized eggs with an iodophor solution (2, 4) may aid in reducing transmission by this route. The second and most likely mode is horizontal transmission, by which infected fish shed virus into the water to infect susceptible fish (21, 32). Low concentrations of IHNV have been detected in waters that contain infected salmonids (6, 21, 23, 29). Avoidance of waterborne exposure to IHNV is a potentially powerful method for preventing the disease in susceptible hatchery stocks.

Iodine is an effective disinfectant against bacteria, viruses, protozoa, and fungi (9, 12). Low levels are generally sufficient to disinfect surfaces, but levels of iodine as high as 1% (10,000 mg/liter) have still not provided complete inactivation of resistant viruses under specific conditions (5, 28). Iodophor disinfectants have been tested for virucidal activity against three fish viruses (4, 10) and for inactivation of selected bacterial and fungal fish pathogens (27). High levels of iodine (25 to 100 mg/liter) are used for disinfection of egg surfaces or hatchery equipment. According to Amend and Pietsch (4), iodine at 12 mg/liter completely inactivated IHNV within 30 s. The goal of the present study was to investigate whether very low levels of elemental iodine could be used as a low-cost water treatment at hatcheries where IHNV is enzootic. Several concentrations of iodine were combined with IHNV under various environmental conditions. Concentrations of iodine were low enough to be nontoxic to rainbow trout (*Oncorhynchus mykiss*) fry but high enough to inactivate IHNV. The results suggest that iodine treatment of hatchery water is a useful method for controlling IHN.

* Corresponding author.

MATERIALS AND METHODS

Cell and virus stocks. Epithelioma papulosum cyprini (EPC) cells (11) were maintained at 25°C in minimum essential medium (MEM; Flow Laboratories, Inc., McLean, Va.) containing 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) and 0.3% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). Addition of 7.5% sodium bicarbonate was used to adjust the pH to between 7.6 and 7.8.

The IHNV isolates used in this study are listed in Table 1. Unless otherwise specified, inactivation trials were conducted with the isolate of IHNV recovered from an adult spawning steelhead trout (*O. mykiss*) at the Dworshak National Fish Hatchery (Ahsahka, Idaho). Stock virus was prepared after three passages on EPC cell monolayers. Culture fluid was clarified by low-speed centrifugation (1,800 × *g*, 10 min) and frozen at −70°C until needed. For each trial, an aliquot of virus was thawed and diluted to 10^5 PFU/ml unless otherwise noted.

Plaque assay. Trypsinized EPC cells were suspended in MEM that contained 0.3% tryptose phosphate broth, 12 mM Tris buffer (pH 7.6, MEM-T), and 5% FBS (MEM-5T). The cells were seeded into eight-well multiplates (Miles Scientific, Naperville, Ill.) at a density of 2×10^6 cells per well and incubated at 25°C overnight. To enhance the sensitivity of EPC cells to infection with IHNV, we followed the cell pretreatment procedure of Batts and Winton (7), in which 100 μl of a 7% polyethylene glycol solution (molecular weight, 20,000; prepared in MEM-5T) was dispensed onto each drained cell monolayer 30 min before inoculating virus.

TABLE 1. Summary of IHNV isolates and characteristics of the five electropherotypes tested for inactivation by iodine

Isolate location	Host species[a]	Yr isolated	No. of cell culture passages	Virus type[b]
Dworshak Hatchery, Idaho	St	1986	4	1
Round Butte Hatchery, Oreg.	St	1985	2	1
Cowlitz Hatchery, Wash.	St	1983	2	2
Sacramento River, Calif.	Ch	1966	?	3
Coleman Hatchery, Calif.	Ch	1985	2	4
Cedar River, Wash.	Soc	1989	3	5

[a] Viruses were all isolated from adult spawning salmonids (St, steelhead trout [*O. mykiss*]; Ch, chinook salmon [*O. tshawytscha*]; Soc, sockeye salmon [*O. nerka*]).

[b] Virus isolates were classified according to electrophoretic mobility of structural proteins (14).

We inoculated 100 μl of each virus dilution onto duplicate monolayers, allowed virus adsorption for 30 min, and then added 2 ml of methylcellulose (0.75%) overlay to each well (8, 19). The infected cells were incubated at 15°C for 7 days to allow plaque formation before they were stained and fixed with a crystal violet-Formalin solution. Plaques were counted, and the mean virus concentration of three replicates was converted to $\log_{10}$ values.

General experimental design. Three replicate treatments were performed for each test. Equal volumes of virus and iodine solutions were reacted for a selected time, and then an equal volume of MEM-T containing 0.1% bovine serum albumin (BSA; MEM-BSA) was added to stop the reaction (unless otherwise specified). A stock solution of 100 mg of free iodine per liter was prepared by dissolving polyvinylpyrrolidone-iodine (PVP-iodine; Sigma Chemical Co., St. Louis, Mo.) in distilled-deionized water (Nanopure II; Barnstead Co., Newton, Mass.). The iodine concentration (reported as milligrams of free iodine per liter) was determined with a chlorine meter (Hach Co., Loveland, Colo.) that had been calibrated with an iodine standard. For each experiment, negative (MEM only) and positive (IHNV only) controls were included, as well as a pair of IHNV inactivation controls containing 0.4 mg of free iodine per liter with or without BSA. Each experiment was required to show virus inactivation for the iodine-IHNV control and no inactivation when iodine was incubated with MEM-BSA before combination with IHNV. The plaque assay was used to determine the number of remaining infectious virus particles.

Iodine efficacy in distilled-deionized water. Iodine and IHNV solutions were prepared in distilled-deionized water, and equal (50-ml) volumes were simultaneously poured into a sterile beaker and mixed with a magnetic stirrer. At 7.5, 15, 30, 60, 120, 240, 480, 960, and 1,920 s, a 1-ml volume was removed from the mixture and added to 1 ml of MEM-BSA to stop the inactivation process. Three replicates of the virus inactivation study were performed at final free iodine concentrations of 0.013, 0.026, 0.056, 0.11, 0.22, and 0.42 mg/liter.

Efficacy of different iodine formulations. Solutions containing powdered PVP-iodine, iodophor (a liquid PVP-iodine solution; Argent Chemical Laboratories, Redmond, Wash.), or pure iodine crystals (Sigma) were prepared in distilled-deionized water to provide a 100-mg/liter stock solution. To compare the ability of the three iodine types to inactivate IHNV, we tested three replicates of each iodine type at 0.10, 0.20, and 0.40 mg of final free iodine per liter. Virus and iodine (50 ml each) were poured into a sterile beaker and mixed by using a magnetic stirrer. A 1-ml sample was removed from the solution after 7.5 s and added to 1 ml of MEM-BSA to stop the inactivation process.

Iodine efficacy against different IHNV isolates. Isolates of IHNV representing each of the five electropherotypes (Table 1) were diluted to 10^5 PFU/ml in 50 ml of distilled-deionized water and combined with an equal volume of PVP-iodine solution to provide a final concentration of 0.10 mg/liter. After 7.5 s of mixing in the reaction beaker, 1 ml was removed and added to 1 ml of MEM-BSA to stop the inactivation process.

To determine whether the concentration of IHNV itself affected the inactivation by iodine, we diluted stocks of the Dworshak isolate in distilled-deionized water to provide final virus stock dilutions of 1:100, 1:1,000, and 1:10,000 (10^6, 10^5, and 10^4 PFU/ml). Equal volumes of virus and PVP-iodine solutions were combined to give final free iodine concentrations of 0.07, 0.25, 0.42, and 0.62 mg/liter. After 5 min of contact, the reaction was stopped by the addition of an equal volume of MEM-5T. To determine the iodine demand of the stock virus suspension, we added an equal volume of iodine solution (0.7 mg/liter, final concentration) to each of the dilutions of virus. After 5 min, the residual free iodine was measured.

Effect of water quality on efficacy of iodine. The effect of water pH, temperature, salinity, and hardness on the ability of iodine to inactivate IHNV was examined. For each experiment, equal volumes (0.5 ml) of the Dworshak isolate of IHNV and PVP-iodine (0.4 mg/liter, final free iodine concentration) were combined for a 5-min exposure followed by the addition of MEM-BSA.

The effect of pH on IHNV inactivation by iodine was tested by using 0.02 M phosphate buffer (pH 6.0, 6.5, 7.0, and 7.5; Sigma) and 0.05 M Tris buffer (pH 7.5, 8.0, 8.5, and 9.0; Sigma) as diluents. Stock solutions (100 mg of free iodine per liter) of PVP-I or pure iodine crystals were diluted to provide a 0.4-mg/liter final free iodine concentration when reacted with IHNV. The titers of virus diluted in buffer solutions were determined at each pH and used as iodine-free controls. Measurements of pH were conducted by using a pH meter suitable for use in Tris buffers (Corning Glass Works, Corning, N.Y.).

The effect of temperature on IHNV inactivation by iodine was tested at 0, 5, 10, 15, 20, and 25°C. Virus and iodine solutions were prepared separately in distilled-deionized water acclimated to each temperature. Distilled-deionized water was combined with IHNV at each temperature to form iodine-free controls.

The effect of salinity on IHNV inactivation by iodine was tested at 0, 4.0, 7.5, 15.5, and 32.0‰. Virus and iodine were prepared in twofold dilutions with filtered seawater obtained from northern Puget Sound, Wash. The salinity was measured with a refractometer. Dilutions of seawater were combined with IHNV to form iodine-free controls.

To compare the effect of water hardness, we prepared solutions by the method of Marking (16), except that 500 mg of sodium bicarbonate per liter was added to control pH and alkalinity. Water hardness and alkalinity were measured with test kits (Hach Co.) and reported as total milligrams of $CaCO_3$ per liter. Iodine and IHNV were prepared with hardness solutions of 0, 10, 20, 40, 80, 160, and 320 mg/liter.

Iodine efficacy in the presence of organic material. The competitive effect of organic material on the ability of iodine to inactivate IHNV was examined. Two proteins, BSA and FBS, were diluted in distilled-deionized water and mixed with equal volumes of IHNV before the addition of iodine.

TABLE 2. Summary of physical characteristics for the water sources used for experimental iodine inactivation of IHNV

Water source	Hardness[a] (mg/liter)	Alkalinity[a] (mg/liter)	pH[b]
Lyons Ferry Hatchery, Wash.	154	160	6.6
Round Butte Hatchery, Oreg.	46	80	7.1
Coleman Hatchery, Calif.	68	70	7.0
Cedar River, Wash.	26	35	6.5
NFRS wetlab, Wash.[c]	42	45	6.7
Dworshak Hatchery, Idaho	14	20	6.0
University of Washington Hatchery, Wash.	43	50	6.8
Soap Lake, Wash.	24	6,900	10.0

[a] Hardness and alkalinity were measured by using test kits (Hach Co.) and expressed as milligrams of $CaCO_3$ per liter.

[b] The hydrogen ion concentration was measured by using a calibrated pH meter (Corning).

[c] Lake Washington water from the wetlab at the National Fisheries Research Center in Seattle was tested as a diluent.

Suspensions of BSA were prepared at 0, 0.004, 0.008, 0.016, 0.032, 0.064, 0.128, 0.256, and 0.512%, and suspensions of FBS were prepared at 0, 0.032, 0.064, 0.128, 0.256, 0.512, 1.024, 2.048, and 4.096%. For each trial, 0.5 ml of each protein suspension was added to 0.5 ml of IHNV (2×10^5 PFU/ml). After 2 min of incubation, 1 ml of 0.8-mg/liter iodine solution was added to the mixture and allowed to react for 5 min. The iodine in the BSA-virus mixtures was neutralized by the addition of 2 ml of MEM-5T, and the iodine in the FBS-virus mixtures was neutralized by the addition of 2 ml of MEM-BSA.

This experiment was repeated with natural sediment from the Cedar River, Wash. Sediment at 0, 0.10, 0.40, 1.60, and 6.40% (wt/vol) was suspended in distilled-deionized water containing IHNV. After a 5-min exposure of iodine to the virus-sediment mixtures, 2 ml of MEM-BSA was added to stop the inactivation process.

Iodine was also combined with several concentrations of BSA for 2 min before adding an equal volume of IHNV. After exposure for 5 min, MEM-5T was added to stop the inactivation process.

Iodine neutralized by sodium thiosulfate. Because removal of free iodine might be required before treated water could be used for fish culture, we tested the concentration of sodium thiosulfate that could neutralize the level of iodine used. Sodium thiosulfate at 0, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M was reacted for 2 min with an equal volume of 0.88-mg/liter (3.5×10^{-6} M) free iodine (PVP-I). An equal volume of IHNV was added to this mixture for a 5-min exposure before the iodine was neutralized with MEM-BSA. Distilled-deionized water was mixed with IHNV for 5 min as an iodine-free control.

Iodine efficacy in natural water samples. Water samples were collected from locations where IHN epizootics had occurred or where water had particular physical characteristics of interest (Table 2). Each water source was used to prepare iodine solutions at several concentrations and to prepare a stock solution of the Dworshak isolate of IHNV. Equal (50-ml) volumes of PVP-iodine and IHNV solutions were poured simultaneously into a sterile beaker and mixed with a magnetic stirrer. A 1-ml sample was removed after 7.5 s of mixing and added to 1 ml of MEM-BSA to stop the inactivation process. The virus inactivation tests were performed for each water source at final free iodine concentrations of 0, 0.05, 0.10, 0.20, 0.40, and 0.80 mg/liter.

Efficacy of pilot plant treatment of hatchery water. At the Dworshak National Fish Hatchery, a saturated solution prepared from bulk-grade crystal iodine was added by a metering pump to provide a 0.31-mg/liter free iodine concentration in water delivered to raceways used for juvenile fish rearing. Three replicates of the following combinations were performed: (i) a 90-ml sample of iodine-treated water was removed from the raceway; (ii) a 45-ml sample of iodine-treated water was removed from the raceway and added to 45 ml of raw hatchery water to provide a 1:2 dilution; (iii) a 22.5-ml sample of iodine-treated water was removed from the raceway and added to 67.5 ml of raw hatchery water to provide a 1:4 dilution. The solutions were mixed and combined with 10 ml of IHNV. After 7.5, 15, 30, and 60 s of mixing on a magnetic stirrer, a 1-ml volume was removed and added to 1 ml of MEM-BSA to neutralize the iodine. As an iodine-free control, 90 ml of raw hatchery water was combined with 10 ml of IHNV. After 60 s, a 1-ml volume was transferred to 1 ml of MEM-BSA.

Acute toxicity and efficacy of iodine. Preliminary fish toxicity and efficacy trials were performed. Iodine concentrations of 0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg/liter were prepared in aquaria supplied with water from Lake Washington. For each iodine concentration, the Dworshak isolate of IHNV was added to provide 10^3 PFU/ml in each of two replicate aquaria, while two other aquaria received cell culture medium. After the virus was exposed for 5 min to iodine, 20 rainbow trout fry were placed in each aquarium for 1 h (with aeration). Flowing water was then provided to all aquaria, and dead fish were removed each day for virus analysis. Whole fry were diluted 1:10 (wt/vol) in MEM-T and homogenized, debris was removed by centrifugation ($1,000 \times g$, 10 min), and the supernatant was assayed by plaque assay. The experiment was terminated after 14 days.

RESULTS

Iodine efficacy in distilled-deionized water. At a final concentration of 0.056 mg of free iodine per liter in distilled-deionized water, a 7.5-s exposure inactivated over 99.99% of IHNV (Fig. 1). Iodine at 0.026 mg/liter and 7.5-s contact inactivated 98.2% of the infectious virus, whereas a 60-s contact was required to achieve 99.99% inactivation. A virus inactivation of 97.2% occurred when 0.013 mg of free iodine per liter was incubated with IHNV for 60 s. As expected, a greater inactivation of virus occurred when the iodine concentration or contact duration was increased. Complete inactivation of virus occurred within 7.5 s at iodine concentrations of 0.11, 0.22, and 0.42 mg of free iodine per liter. Virus inactivation was not appreciably greater when exposure times increased from 2 to 32 min.

Efficacy of different iodine formulations. Powder, liquid (iodophor), and crystal iodine at 0.10 mg/liter provided greater than 99.99% reduction of IHNV titers in a 7.5-s exposure. Although the majority of the laboratory studies were done with PVP-I as a powder, the results were comparable to those achieved using iodine from crystals, the form likely to be used in treatment of hatchery water due to cost, commercial availability, and ease of application.

Iodine efficacy against different IHNV isolates. Virus isolates representing the five electropherotypes of IHNV were inactivated by at least 99.4% by exposure for 7.5 s to iodine at a final concentration of 0.10 mg/liter (Table 3). At this short contact time and low level of iodine, isolates of IHNV belonging to electropherotype 1 or 2 were inactivated to a greater extent.

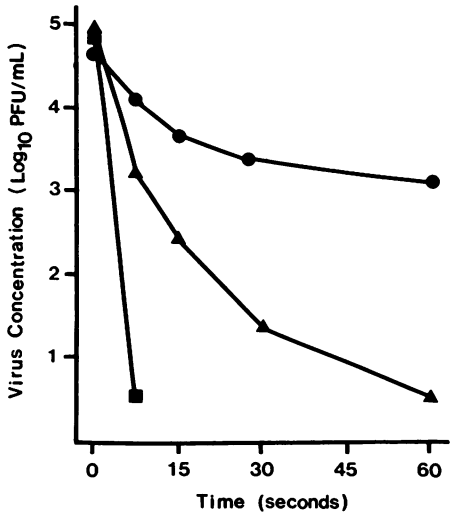

FIG. 1. Inactivation of the Dworshak isolate of IHNV when combined with three iodine concentrations for various contact times. Virus and iodine solutions were prepared in distilled-deionized water and mixed with a magnetic stirrer. At each time point, a sample was removed and combined with a solution of BSA to stop the inactivation process. The detection limit of the plaque assay was 0.52 $\log_{10}$ PFU/ml. The free iodine concentrations after combining with virus were 0.013 mg/liter (●), 0.026 mg/liter (▲), and 0.056 mg/liter (■).

Changes in the dilution of stock virus resulted in quite different inactivation efficiencies. When the Dworshak isolate was combined for 5 min at 0.07 mg of free iodine per liter, over 99.9% of IHNV was inactivated at stock virus dilutions of 1:1,000 or 1:10,000. However, inactivation was relatively poor (<97.2%) at free iodine concentrations up to 0.62 mg/liter when the virus was diluted 1:100. When iodine (0.7 mg/liter, final concentration) was added to stock virus dilutions of 1:100, 1:1,000, and 1:10,000 (final), the free iodine concentration remaining was less than 10% (below detection), 41%, and 94%, respectively.

Effect of water quality on efficacy of iodine. Inactivation of IHNV by iodine showed a reduction in efficacy with increasing pH (Table 4). When reacted for 5 min at 0.4 mg of free iodine per liter in water buffered at pH 6.0, 6.5, 7.0, and 7.5, the titer of infectious virus was reduced by at least 99.95%. For higher pH levels, the efficacy continued to drop to about 90% reduction at pH 9.0. During preliminary experimental trials, several biological buffers neutralized free iodine rapidly and could not be used for inactivation studies (data not shown).

TABLE 3. Inactivation of selected virus isolates representing the five electropherotypes of IHNV by 0.10 mg of iodine (PVP-I) per liter in distilled-deionized water

IHNV isolate	Strain type[a]	Virus titer ($\log_{10}$ PFU/ml)		% Reduced
		Water + IHNV[b]	Iodine + IHNV[c]	
Dworshak	1	4.62	<0.52	>99.99
Round Butte	1	5.11	1.70	99.96
Cowlitz	2	4.53	<0.52	>99.99
Sacramento River	3	5.18	2.88	99.49
Coleman	4	5.00	2.34	99.78
Cedar River	5	5.00	2.18	99.85

[a] Virus isolates were classified according to Hsu et al. (14). See Table 1 for details about the isolates used.
[b] Water and IHNV were combined in 50-ml volumes for 60 s before a sample was added to MEM-BSA.
[c] Iodine and IHNV were combined in 50-ml volumes for 7.5 s before a sample was added to MEM-BSA to stop the inactivation process.

TABLE 4. Effect of pH on the inactivation of the Dworshak isolate of IHNV by 0.4 mg of iodine per liter as PVP-I or pure crystals[a]

Buffer	pH	Mean virus titer ($\log_{10}$ PFU/ml)		% Reduced[b]	Mean virus titer ($\log_{10}$ PFU/ml) of crystal + IHNV	% Reduced[b]
		Buffer + IHNV	PVP-I + IHNV			
Phosphate	6.0	4.62	<0.52	>99.99	<0.52	>99.99
Phosphate	6.5	4.52	<0.52	>99.99	<0.52	>99.99
Phosphate	7.0	4.66	<0.52	>99.99	<0.52	>99.99
Phosphate	7.5	4.70	<0.52	>99.99	<0.52	>99.99
Tris	7.5	4.72	0.83	99.99	1.43	99.95
Tris	8.0	4.74	1.80	99.89	2.15	99.75
Tris	8.5	4.69	2.32	99.57	2.63	99.12
Tris	9.0	4.71	3.67	90.78	3.76	88.63

[a] Virus and iodine were diluted in buffered solutions and combined for 5 min before the inactivation process was stopped with MEM-BSA.
[b] Percent reduction of virus titer by iodine compared with controls containing buffer and virus.

Inactivation of IHNV by iodine was not affected by temperature under the conditions used (data not shown). When incubated for 5 min at 0.4 mg of iodine per liter, the titer of infectious virus was reduced by at least 99.95% for all temperatures tested.

Inactivation of IHNV by iodine was not affected by salinity under the conditions used (data not shown). The titer of infectious virus was reduced by at least 99.99% when incubated for 5 min at 0.4 mg of iodine per liter in dilutions of natural seawater containing 0, 4.0, 7.5, 15.5, and 32.0‰ salts.

Inactivation of IHNV by iodine was not greatly affected by water hardness as long as the alkalinity and pH were controlled (data not shown). Iodine at 0.4 mg/liter inactivated IHNV in 5-min exposures by at least 99.8%. In this study, pH was maintained between 7.4 and 7.8 and alkalinity was maintained between 257 and 274 mg/liter as $CaCO_3$.

Iodine efficacy in the presence of organic material. The proteins BSA and FBS efficiently prevented the inactivation of IHNV by iodine. When ≤0.016% BSA (final) was combined with the virus before adding the iodine, the virus titer was reduced at least 99.96%. However, at ≥0.032% BSA (final), the IHNV titer reduction was less than 93% (Table 5). A final BSA concentration of 0.128% had no effect on the detection of virus by the plaque assay.

When 0.4 mg of final iodine per liter was combined with BSA for 2 min before adding IHNV, the inactivation by iodine was poor at 0.0080% BSA (final concentration with iodine added) but excellent at a BSA concentration of 0.0040% or lower (Table 5).

FBS was less effective than BSA at blocking IHNV inactivation by iodine (data not shown). When ≤0.128% (final) FBS was combined with the virus before adding the iodine, the virus titer was reduced at least 99.99%. However, at ≥0.256% (final), the IHNV titer reduction was less than 97.5%. A final FBS concentration of 6.4% had no effect on the detection of IHNV by the plaque assay.

When ≤0.40% (final) Cedar River sediment was combined

TABLE 5. Effect of BSA on iodine (PVP-I) inactivation of the Dworshak isolate of IHNV[a]

BSA[b] (%)	Iodine[c] (mg/liter)	Iodine + BSA + IHNV		IHNV + BSA + iodine	
		Titer[d]	% Reduced	Titer	% Reduced
0	0	5.04		4.81	
0	0.4	<0.52	>99.99	<0.52	>99.99
0.002	0.4	<0.52	>99.99	<0.52	>99.99
0.004	0.4	0.83	99.99	<0.52	>99.99
0.008	0.4	4.81	41.82	<0.52	>99.99
0.016	0.4	5.04	0.00	1.11	99.96
0.032	0.4	ND[e]	ND	3.41	92.78
0.064	0.4	ND	ND	4.08	66.67

[a] Equal volumes (500 μl) of the first solutions were combined for 2 min before 1,000 μl of the other solution was added. After 5 min, MEM-5T was added to stop the inactivation process.
[b] BSA was prepared in distilled-deionized water. The level of BSA reported is the concentration after combination with iodine.
[c] Final iodine concentration after being combined with IHNV.
[d] Remaining IHNV (log_{10} PFU/per milliliter) by plaque assay.
[e] ND, not done.

with IHNV before adding iodine, the titer of IHNV was reduced by greater than 99.99% (data not shown). At 1.60% (final) sediment, the titer of IHNV was reduced by 99.75%, suggesting that little, if any, reduction in efficacy was caused by sediment levels that exceed those normally encountered in natural water supplies. No virus reduction was attributed to the presence of sediments in controls lacking iodine.

Iodine neutralized by sodium thiosulfate. A low concentration of sodium thiosulfate effectively neutralized the ability of iodine to inactivate IHNV (data not shown). The control virus titer without iodine present was 4.54 log_{10} PFU/ml. When 10^{-2}, 10^{-3}, 10^{-4}, or 10^{-5} M sodium thiosulfate was added to an equal volume of 0.88-mg/liter (3.5×10^{-6} M) iodine before combining with IHNV, no virus titer reduction occurred. However, at 10^{-6} M, a 99.99% titer reduction occurred, suggesting that insufficient sodium thiosulfate was now present to neutralize the iodine.

Iodine efficacy in natural water samples. When virus and iodine were prepared in water samples from various natural sources and mixed for 7.5 s, 0.20 mg of iodine per liter inactivated 99.9% of the IHNV for six of the eight water sources (Table 6). The best natural diluent water for iodine inactivation of IHNV was from the Lyons Ferry hatchery; 0.10 mg of iodine per liter inactivated 99.98% of the IHNV and 0.05 mg of iodine per liter inactivated 95.0% when diluted in this water. A concentration of 0.20 mg of iodine per liter inactivated 97.3% of the virus when water from the University of Washington hatchery was used as the diluent, although 0.40 mg of iodine per liter reduced the titer by greater than 99.99%. An iodine concentration of 0.80 mg/liter was required before any significant inactivation of IHNV was observed in Soap Lake water.

TABLE 6. Remaining IHNV (Dworshak isolate) concentration following 7.5-s exposure to several iodine (PVP-I) concentrations with various water sources as the diluent[a]

Water source as diluent[b]	Mean titer (log_{10} PFU/ml) at a final iodine concn (mg/liter) of:					
	0	0.05	0.10	0.20	0.40	0.80
Lyons Ferry	4.91	3.61	1.23	<0.52[c]	ND[d]	<0.52
Round Butte	4.79	4.36	2.74	0.52	0.52	<0.52
Coleman	4.40	4.36	3.32	<0.52	<0.52	<0.52
Cedar River	4.70	4.79	3.45	<0.52	0.52	<0.52
NFRS wetlab	4.54	4.54	4.51	0.83	<0.52	<0.52
Dworshak	4.82	4.88	4.87	1.52	<0.52	<0.52
University of Washington	4.41	4.45	4.46	2.85	<0.52	<0.52
Soap Lake	4.63	4.81	4.86	4.57	4.18	0.52

[a] The iodine was neutralized by adding the mixture to an equal volume of MEM-BSA.
[b] See Table 2 for details of physical characteristics of each water source.
[c] Detection limit of IHNV by the plaque assay was 3.3 PFU/ml.
[d] ND, not done.

TABLE 7. Inactivation of the Dworshak isolate of IHNV when mixed with samples of Dworshak Hatchery (Idaho) water metered continuously to contain 0.31 mg of free iodine per liter[a]

Treatment + IHNV	Final iodine (mg/liter)[b]	Mean IHNV titer (log_{10} PFU/ml) after a treatment exposure of:			
		7.5 s	15 s	30 s	60 s
Raw water	0	ND[c]	ND	ND	4.46
Iodinated	0.28	0.52	<0.52	<0.52	<0.52
Iodinated	0.14	1.48	0.83	0.52	0.52
Iodinated	0.07	4.54	4.51	4.46	4.43

[a] Following exposure of virus to dilutions of iodine-treated water, the inactivation process was stopped by adding MEM-BSA.
[b] Iodine concentration was determined from test kit results (Hach Co.) and dilution of iodine-treated water with IHNV. Twofold dilutions of the iodine-treated water were performed with Dworshak raw hatchery water as the diluent.
[c] ND, not done.

Efficacy of pilot plant treatment of hatchery water. Samples of iodine-treated hatchery water effectively inactivated IHNV. When 0.28 mg of iodine per liter (final concentration) was reacted with IHNV for 7.5 s, the virus titer was reduced by 99.99% (Table 7). At 0.14 mg of iodine per liter, a 7.5-s contact with virus resulted in 99.90% inactivation, whereas a 30-s contact inactivated 99.99% of the virus. No reduction in virus titer was evident at 0.07 mg of iodine per liter for exposure times up to 60 s.

Acute toxicity and efficacy of iodine. Preliminary tests were conducted to determine the ability of iodine to prevent infection of fish and to test the acute toxicity of iodine for rainbow trout fry (data not shown). Fish were not infected following exposure to a solution of iodine (≥0.25 mg/liter, final concentration) combined with 10^3 PFU of IHNV per ml, whereas IHN mortality (42.5%) occurred in fish receiving IHNV alone. Mortality (100%) from acute toxicity occurred within 24 h when fish were placed in rearing water containing 2 and 4 mg of free iodine per liter, but no iodine-associated mortality was observed at or below 1 mg of iodine per liter. No measurable iodine remained after 1 h in the aquaria containing fish, possibly due to the iodine binding to organic wastes excreted by the fish or to the fish themselves.

DISCUSSION

This study examined factors affecting the inactivation of IHNV in water by low levels of iodine. Under the conditions used in this study, water temperature, salinity, and hardness had no effect on the inactivation. Because IHNV is a salmonid fish virus, we did not test the inactivation process at temperatures, salinities, pHs, or hardness levels outside the range encountered by the virus in nature. All iodine formulations effectively inactivated the strains of IHNV that were used.

The inactivation of IHNV by iodine was significantly affected by the pH of the solution. When virus and iodine were reacted at various pH levels, inactivation decreased with increasing pH. This suggests that for waters having higher pH, the iodine concentration may need to be increased somewhat to obtain sufficient inactivation of IHNV. Iodine prepared in Soap Lake water (pH 10.0) was effective only when 0.8 mg of free iodine per liter was present, although this water was outside the pH range (6.5 to 8.0) at which trout normally are reared (26).

Under optimal conditions, a concentration of 0.056 mg of iodine per liter reduced the IHNV titer by 99.99% in 7.5 s. When Amend and Pietsch (4) tested two iodophor disinfectants against salmonid viruses, they reported that a 30-s exposure to 12 mg of iodine per liter or a 15-s exposure to 25 mg of iodine per liter was required for complete inactivation of IHNV. The higher level of iodine reported in that study might reflect differences in the amount of virus and total protein present in the reaction mixture. Our results showed that the efficacy of iodine was dependent on the dilution of the IHNV stock. This difference was probably due to the presence of additional organic material (e.g., FBS or debris from infected cell cultures) in the less dilute preparation. Also, the high virus titer itself may have exerted an iodine demand.

Horizontal (waterborne) transmission may constitute a major route by which young fish are exposed to IHNV (21). Prevention of exposure of susceptible salmonids to IHNV requires an effective mechanism for reducing the amount of virus coming into a hatchery. For some hatcheries, waterborne exposure can be eliminated with pathogen-free water from wells or springs. Other hatcheries require treatment of the water to remove pathogens, including viruses. In this regard, ozone treatment of hatchery water has received attention (30, 31).

Although ozone is a good oxidizer and is effective at killing fish pathogens, it is toxic to fry at low concentrations (31) and must be removed before fish are exposed to the treated water. The biggest drawback to ozone treatment is the cost of equipment and operation. To be fail-safe, a backup system is required to disinfect the water in the event of a mechanical or electrical failure. Even if iodine treatment of the incoming water were not selected as the primary treatment option, it could serve as a backup system to ozone.

Previous methods for prevention and control of IHN either have not been adequately tested at a large-scale salmonid production facility or have failed to provide satisfactory protection. Increasing the water temperature successfully controlled IHN in sockeye salmon (*Oncorhynchus nerka*) juveniles (1) but not rainbow trout (13). To resist viral disease, the host must rely on specific immunity, nonspecific resistance factors (3, 17, 24), or other mechanisms. Various vaccine regimens have been tested (15) and are effective only if fry have reached a sufficient age for immune competence. If susceptible salmonids lack specific immunity, IHN mortality usually occurs soon after hatching (18, 22), and total mortality may reach over 90% of the population. Iodine treatment of hatchery water could protect juvenile salmonids from exposure to IHNV until they reach sufficient size to make vaccination effective.

Iodine treatment of hatchery water may prevent exposure of susceptible fish to IHNV, but higher concentrations of iodine may be required at locations having water with a large organic load or higher pH. In such instances, iodine could be added to the hatchery intake line and removed before delivery to the rearing units. In this study, low concentrations of sodium thiosulfate effectively removed free iodine from the water, suggesting that it could be added to the treatment procedure at low cost.

When IHNV was added to a water sample from a nursery tank receiving an iodine solution at the Dworshak National Fish Hatchery, low levels of iodine rapidly reduced the virus concentration. In these nursery tanks, no mortality was observed among groups of rainbow trout fry continuously exposed for several weeks to an iodine concentration of 0.25 mg/liter. This suggests that levels of iodine could be added to the hatchery water supply that would effectively inactivate IHNV and yet be nontoxic to fish, without requiring removal of the iodine. Inactivation of IHNV was also rapid (7.5 s) when iodine and virus were prepared in water samples from different hatchery supplies, suggesting that the method is broadly applicable.

To ensure that iodine does not induce chronic toxicity, extended contact experiments should be performed. These toxicity studies must incorporate various water quality factors, especially pH and water hardness. Inactivation of IHNV should be tested at each hatchery where this system might be installed.

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