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Effects of Estuarine and Marine Waters on the Infectivities of Infectious Hematopoietic Necrosis Virus (IHNV) and Infectious Pancreatic Necrosis Virus (IPNV)

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Abstract

Brackish and sea waters were collected from estuarine and coastal areas and their comparative capabilities to reduce the infectivity titres of infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) were evaluated.

Significant titre reduction of IHNV was observed within 3 days of post-incubation in both untreated brackish and sea water samples and sediment-contained water samples. The titre reduction to less than detectable limits was also found even at 0 time (within 1 hour after the virus was inoculated) in these untreated samples. The infectivity of IPNV was reduced approximately 99% to 99.9% by untreated sea water and the sediment-contained water during a 14-day-incubation period. The reduction of the infectivity was more rapid in seawater than brackish water. However, loss of infectivity was little noted for both viruses in bacteria-free water treated by filtration or autoclaving. Observed results were similar in all water collected in each of the four seasons.

These phenomena of loss of virus infectivity might be profoundly related to bacterial activity in the samples since viable bacterial counts significantly increased during the incubation period. Moreover, the fact that IHNV was inactivated by pure bacterial cultures of *Achromobacter* sp. and *Pseudomonas* sp. isolated from the brackish water reinforces this hypothesis.

Introduction

Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV) are fish viruses which cause significant losses of salmonid fishes throughout the world (Pilcher and Fryer, 1980). So far, no methods for preventing outbreaks of disease caused by these viruses have been established and preventing measures are therefore urgently needed.

It is known that virus infectivity is affected by physical (Amend, 1970; Lo et al., 1976; Mackelvie and Desautels, 1975; Pietsch et al., 1977; Yates et al., 1985), chemical (Desautels and Mackelvie, 1975; Lo et al., 1976; Mackelvie and Desautels, 1975; Pietsch et al., 1977; Salo and Cliver, 1978; Ward and Ashley, 1977) and biological factors (Cliver and Herrmann, 1972; Labelle et al., 1980; Richards et al., 1978; Toranzo and Hetrick, 1982; Toranzo et al., 1982, 1983a, 1983b). Several investigators have reported antiviral activity in sea water (Gerba and Schaiberger, 1975; Lo et al., 1976; Matossian and Garabedian, 1967; Mitchell, 1971). Magnusson et al. (1967) concluded that virus inactivation in sea water was attributed to

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marine bacteria or substances from the bacteria and that the causative bacterium was *Vibrio marinus*. Fujioka et al. (1980) observed that enteroviruses were inactivated in untreated seawater, but not in the seawater treated by boiling, autoclaving, or filtration, suggesting that microbial activity was responsible for this effect. Similar findings have been proven using IPNV in estuarine water by Toranzo et al. (1983b).

In contrast, some researchers have reported that enterovirus survival was prolonged in water containing sediment (Smith et al., 1978). In a previous paper, we demonstrated that the raceway water of a salmonid hatchery inactivated IHNV and some particular bacteria, such as *Pseudomonas*, played a part in virus inactivation (Yoshimizu et al., 1986).

In the present study, water and sediments were collected from estuarine and marine environments during each of the four seasons from 1984 to 1985 and the effect of those samples on IHNV or IPNV inactivation was examined, comparing water quality, seasonal variation, and changes in bacterial counts between pre- and post-incubation.

Materials and Methods

Cell cultures and viruses

The rainbow trout (*Salmo gairdneri* Richardson) gonad cell-line, RTG-2 (Wolf and Quimby, 1962), was used for replication and titration of both IHNV and IPNV. Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells, CHSE-214 (Fryer et al., 1965), was also used for plaque assay. IHNV (strain ChAb) was isolated from chum salmon (*Oncorhynchus keta*) in our laboratory (Kimura et al., 1981) and IPNV (strain VR299) was obtained from Dr. J.L. Fryer (Oregon State University, USA). The viruses were propagated in RTG-2 cells grown in 75 cm^2-plastic flasks (Falcon) containing 25 ml of MEM10-Tris medium (pH : 7.2) : Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (M.A. Bioproduct), 0.075% $NaHCO_3$, 100 IU/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma), and 1.6% Tris-buffer (Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride) (Sigma) adjusted to pH 7.8. When the cytopathic effect was complete, the culture fluid was removed from the flask and centrifuged at 2,100 $\times$ g at 4° C for 20 min. The supernatant was passed through a 0.45 μm pore-sized membrane filter (Millipore) and stored at −80°C until used.

Virus assays

Virus assays for studies of virus survival in environmental water were performed by the $TCID_{50}$ method using a 96-well microtitre plate (Falcon). Serial 10-fold dilutions of virus were made using Hanks' balanced salt solution (Harks' BSS, Gibco), supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.0375% $NaHCO_3$. Each of 4 wells was inoculated with 0.05 ml of each virus dilution. After incubation at 15°C for 10 days, the cells were fixed with 10% formalin, stained with 0.1% crystal violet, and then measured for cytopathic effect. The $TCID_{50}$ was calculated by the method of Kärber (1931).

Water collection

Seasonal water samples were collected in June, August, and October, 1984 and January, 1985. Samples were collected in sterile glass bottles from the surface at two sampling sites: brackish water from the estuary of the Moheji river near Hakodate, Japan and seawater from the Nanaehama coast near Hakodate, Japan. Sediments were collected in sterile petri dishes from the same sampling sites. Water and sediment samples were transported in cooled bags and used within three hours after collection.

Environmental analysis of sampling sites

Water temperature, pH, turbidity, dissolved oxygen, and conductivity were measured with a water checker (U-7, Horiba Ltd.) at the sampling time. Viable bacterial counts in the water and sediment were enumerated by standard plate count using freshwater agar, FWA (Yoshimizu et al., 1976) or seawater agar, SA medium (Yamamoto et al., 1982). SA and both FWA and SA media were used for saline and brackish water and sediment samples to enumerate the viable bacterial counts respectively.

Virus survival studies

Ninety-nine ml aliquots of the water samples were dispensed into sterile 125 ml-glass bottles. From each location, one sample was sterilized by filtration through a 0.22 μm pore-sized membrane filter (Millipore), another sterilized by autoclaving at 121°C for 15 min, and the third was left untreated. Sediment preparations were made by mixing 1 g of sediment with 98 ml of untreated water. Bottles containing 99 ml of Hanks' balanced salt solution were used for the control. To each bottle, 1 ml of the same lot of virus suspension (5.80 log $TCID_{50}$/ml of IHNV and 8.30 log $TCID_{50}$/ml of IPNV) was added and held at 15°C for 14 days. At 0 time (within 1 hour after virus inoculation), 3, 7, and 14 days after incubation, a 1 ml-aliquot of each sample was withdrawn for titration by $TCID_{50}$ method. The untreated and sediment-contained water samples required filtration through a 0.45 μm pore-sized membrane filter (Millipore) to eliminate bacterial and fungal contaminants before titration. Viable bacterial counts were determined at day 0 and 14. Virus survival was expressed by dividing the virus titre at each sampling time (Nt) by the initial titre of virus (NO).

Identification of bacteria

Each of 30 bacterial strains isolated from water and sediment samples, which were collected seasonally from 1984 to 1985 from the estuary of the Moheji river and the Nanaehama coast and used for viral survival studies at day 0 and 14 after incubation, were identified to genus level by the method of Shewan et al. (1960). A fermentative property of marine bacteria was examined by using the method of Leifson (1963). Bacterial floras of pre- and post-incubation were compared for both the water and sediment samples.

Plaque assay

At the end of incubation of water sample collected from the estuary of the

Moheji river on June 16, 1984, 28 bacterial strains were isolated from the water sample and evaluated for their IHNV inactivation ability. The test strains were inoculated to 50 ml MCYG broth medium (pH : 7.8) containing 0.5% casamino acids (Difco), 0.05% yeast extract (Difco), 0.1% glucose, and 50% Herbst artificial seawater (ASW) in 100 ml-Erlenmeyer's flask and incubated at 20°C for 2 days agitating at 160 r.p.m. The mixture of 0.2 ml of the culture filtrate and the equal volume of IHNV dilution (approximately 200 PFU/0.1 ml) was reacted at 15°C for 3 hours. A 0.2 ml mixture each was inoculated to 2 wells containing confluent monolayer of CHSE-214 cells drained in 24-well microplate (16 mm in diameter, Falcon) and 1 ml of 0.8% methylcellulose overlay medium (Kamei et al., 1987) was added to the cell culture after the virus was adsorbed for 1 hour. After 7-day-incubation at 15°C, the cells were fixed and stained as described above, and the plaques formed were counted. The plaque reduction rate was calculated by comparing with control which was reacted with the fresh broth.

Survival of IHNV in pure bacterial cultures

Two representative bacterial strains, 46BBW-09 and 46BBW-20, which showed plaque reduction of IHNV more than 90% by the cultural filtrates, were examined for actual IHNV inactivation in the pure cultures. Three-day-old bacterial cultures grown in MCYG broth were centrifuged and the cell pellets obtained were rinsed three times with 50% ASW. The bacterial cells were suspended in 99 ml of 50% ASW to give 10^2 cfu/ml (7.0×10^2 cfu/ml, 46BBW-09 ; 7.4×10^2 cfu/ml, 46BBW-20). One ml of stock virus (1.1×10^6 PFU/ml of IHNV) was added to the pure bacterial cultures in 50% ASW and incubated at 15°C for 7 days. At 0, 3, 5, and 7 days after incubation, 1.5 ml-aliquot was withdrawn from the cultures for titration by plaque assay and viable bacterial counts. The bacterial growth in the cell cultures after inoculation was suppressed by adding with 500 μg/ml of kanamycin.

Results

Water and sediment samples from estuarine and coastal environments were collected in each of the four seasons from 1984 to 1985 and evaluated for their ability

Table 1. Environmental conditions of sampling stations.

Station	Date	Water Temp. (°C)	pH	Turb.*	Dissolved Oxygen	Cond.**
Estuary of the Moheji River	6/16, '84	14.9	8.2	7	8.8	25.3
	8/24, '84	16.5	8.2	84	ND	10.7
	10/31, '84	11.6	7.7	8	ND	26.3
	1/28, '85	2.0	6.2	10	7.6	12.8
Nanaehama Coast	6/29, '84	16.1	7.8	7	6.1	38.0
	8/24, '84	21.7	6.6	29	ND	46.1
	10/31, '84	10.7	7.6	ND	ND	35.3
	1/28, '85	3.4	6.3	10	11.2	29.7

*Turb., Turbidity ; **Cond., Conductivity ; ND, not determined.

Table 2. Viable bacterial counts in water and sediments collected in four seasons and in the bottles containing the water and the water-sediment mixture samples at 14 days after incubation.

Station	at Collection Time		After Incubation	
	Water	Sediment	Water	Mixture
Estuary of the Moheji River				
6/16, '84[a]	2.8[b](4.1)[c]	2.8 (3.2)	ND (6.1)	ND (6.2)
8/24, '84	3.6 (3.8)	1.4 (3.4)	6.1 (5.3)	5.7 (6.0)
10/31, '84	3.0 (2.8)	1.8 (1.9)	6.1 (6.4)	6.1 (6.5)
1/28, '85	3.2 (2.4)	2.3 (1.0)	5.5 (5.9)	6.8 (6.4)
Nanaehama Coast				
6/29, '84	(5.1)	(4.4)	(6.2)	(6.2)
8/24, '84	(4.0)	(3.9)	(5.8)	(6.1)
10/31, '84	(4.7)	(3.7)	(6.0)	(6.2)
1/28, '85	(3.1)	(2.9)	(5.6)	(6.4)

[a]: sampling dates in four seasons; [b]: viable bacterial counts on FWA medium for freshwater samples; [c]: viable bacterial counts on SA medium for seawater samples. All the CFU numbers are expressed as logarithmic values per ml of water or 0.01 g of sediment. ND: not determined.

to inactivate viruses. The environmental conditions for each sampling period are listed in Table 1. The reason why turbidities of both sampling stations were much higher in Aug., 24th, 1984 than other dates may be the affect of a typhoon. Viable bacterial counts of water and sediment samples of pre- and post-incubation are shown in Table 2. Estuarine water showed higher viable counts on SA medium than on FWA medium for the samples of spring and summer. After 14-day incubation, the viable counts of the water and sediment samples from the estuary increased 10^2 to 10^4. However, the bacterial numbers of seawater samples from the coast increased only 10^2 during the incubation.

Reduction of infectivity titre of IHNV in estuarine and marine waters is shown in Table 3. Loss of the virus infectivity was conspicuous after incubation and rapid titre reduction was observed even at 0 time when the virus was suspended in untreated samples from both estuarine and marine environments. Water sterilized by filtration and autoclaving allowed the virus to maintain the infectivity. These phenomena were found in all samples collected in each of four seasons.

The titre reduction in the environmental waters for IPNV is shown in Table 4. In the case of IPNV, loss of infectivity was not as rapid as IHNV. However, incubation of 14 days caused the infectivity reduction more than 100 fold and the reduction rate was higher in sea water samples than in brackish water. As similar as the results obtained in IHNV test, cell-free water treated by filtration and autoclaving also did not reduce IPNV infectivity with the exception of water collected from the Nanaehama coast in January, 1985. These selected data are plotted as Figs. 1 and 2. Comparative bacterial floras in water and sediment samples of pre- and post-incubation for each collection season are indicated in Figs.

Table 3. Infectivity (log $TCID_{50}$/ml) of IHNV held in brackish and sea water at 15°C for 14 days.

Sample	Treated				
Date	Untreated	Sediment	Autoclaved	Filtered	Hanks' BSS
6/16, '84					
Moheji River*					
0 (days)	2.80	2.80	4.80	4.55	4.55
3	2.80	2.80	4.55	4.55	4.30
7	2.80	2.80	4.30	3.80	4.80
14	2.80	2.80	2.80	3.05	4.55
6/29, '84					
Nanaehama Coast**					
0 (days)	2.80	3.55	3.80	3.80	4.05
3	2.80	2.80	4.80	4.05	4.80
7	3.05	2.80	3.80	3.80	4.30
14	2.80	4.30	3.30	3.80	3.80
8/24, '84					
Moheji River					
0 (days)	2.80	2.80	4.05	3.80	3.80
3	2.80	2.80	5.30	3.30	4.55
7	2.80	2.80	4.55	3.55	3.55
14	2.80	2.80	3.80	3.05	3.80
8/24, '84					
Nanaehama Coast					
0 (days)	3.05	2.80	3.55	3.05	4.05
3	2.80	3.30	3.55	3.05	4.55
7	2.80	2.80	4.05	3.80	3.05
14	2.80	2.80	3.30	3.80	3.30
10/31, '84					
Moheji River					
0 (days)	2.80	2.80	5.30	5.30	5.55
3	2.80	2.80	5.30	5.05	4.80
7	2.80	2.80	5.55	4.55	5.55
14	2.80	2.80	4.55	3.05	4.55
10/31, '84					
Nanaehama Coast					
0 (days)	3.05	2.80	5.30	4.80	5.55
3	2.80	3.05	5.05	4.30	4.80
7	2.80	3.05	4.80	3.30	5.55
14	2.80	2.80	4.55	2.80	4.55
1/28, '85					
Moheji River					
0 (days)	2.80	2.80	5.80	6.05	5.55
3	2.80	2.80	6.05	5.55	5.55
7	2.80	2.80	6.05	5.30	5.80
14	2.80	2.80	5.80	5.30	5.30
1/28, '85					
Nanaehama Coast					
0 (days)	2.80	2.80	5.55	5.05	5.55
3	2.80	2.80	5.05	5.55	5.55
7	2.80	2.80	4.55	3.80	5.80
14	2.80	2.80	2.80	2.80	5.30

*: brackish water; **: sea water.

Table 4. Infectivity (log $TCID_{50}$/ml) of IPNV held in brackish and sea water at 15°C for 14 days.

Sample	Treated				
Date	Untreated	Sediment	Autoclaved	Filtered	Hanks' BSS
6/16, '84					
Moheji River*					
0 (days)	8.30	8.30	8.80	8.80	8.05
3	7.55	8.30	8.30	8.80	8.80
7	6.80	8.05	8.05	8.05	8.05
14	6.05	7.80	8.05	8.55	8.30
6/29, '84					
Nanaehama Coast**					
0 (days)	7.30	8.30	8.55	8.30	8.55
3	6.80	7.80	8.80	8.55	8.30
7	6.05	7.80	7.80	8.80	8.30
14	5.80	5.80	8.05	8.55	8.55
8/24, '84					
Moheji River					
0 (days)	6.80	6.80	7.80	6.80	7.30
3	6.55	7.05	7.80	6.55	6.80
7	6.55	5.05	6.80	6.55	6.80
14	6.30	6.05	7.30	6.80	7.05
8/24, '84					
Nanaehama Coast					
0 (days)	7.05	5.80	6.80	6.80	7.55
3	6.30	6.30	7.30	7.30	7.05
7	4.80	4.30	6.55	6.55	6.30
14	4.05	3.55	7.05	7.30	6.55
10/31, '84					
Moheji River					
0 (days)	8.05	7.30	8.30	8.80	8.80
3	8.05	8.30	8.30	8.05	8.80
7	8.05	7.55	8.55	8.80	7.30
14	7.55	6.30	8.55	8.55	7.80
10/31, '84					
Nanaehama Coast					
0 (days)	8.05	7.05	8.80	8.30	8.80
3	7.80	8.30	8.05	8.30	8.80
7	4.55	5.80	8.05	8.05	7.30
14	4.80	5.55	8.05	8.55	7.80
1/28, '85					
Moheji River					
0 (days)	5.80	5.55	8.55	7.80	8.55
3	6.05	4.80	8.30	8.80	8.30
7	4.55	5.80	8.05	8.55	7.80
14	6.30	5.55	8.30	8.55	8.55
1/28, '85					
Nanaehama Coast					
0 (days)	3.80	3.80	8.05	8.55	8.55
3	2.80	2.80	7.80	7.55	8.30
7	3.80	3.55	7.05	7.30	7.80
14	4.30	3.55	7.30	5.80	8.55

*: brackish water; **: sea water.

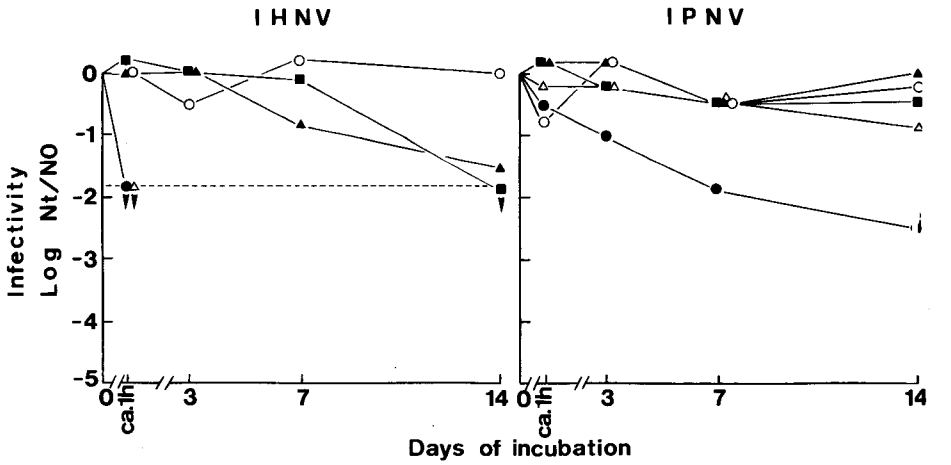

Fig. 1. Infectivities of fish pathogenic viruses in brackish water and water-sediment mixture samples from the estuary of the Moheji river in June, 1984.
Symbols : ■, autoclaved ; ▲, filtered ; ●, untreated ; △, water-sediment mixture ; ○, control. Dotted line and arrows indicate detectable limits of viral tires and less than the limits respectively.

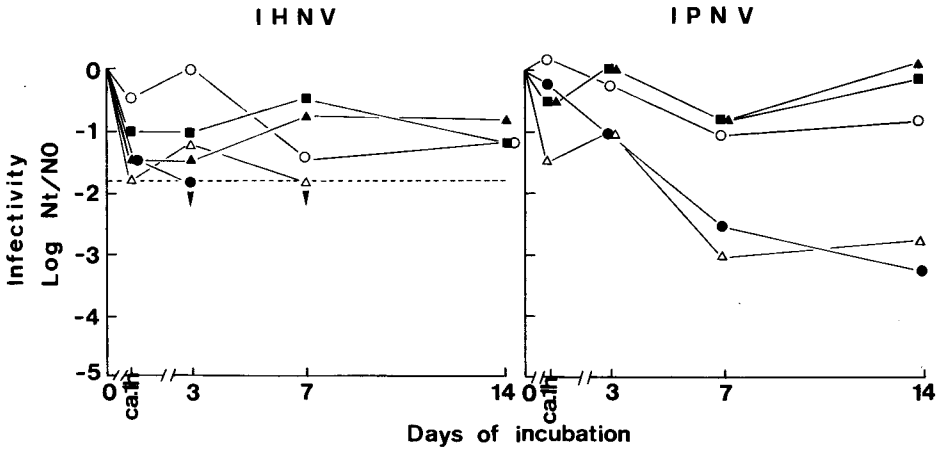

Fig. 2. Infectivities of fish pathogenic viruses in seawater and water-sediment mixture samples from the Nanaehama coast in August, 1984.
Symbols : ■, autoclaved ; ▲, filtered ; ●, untreated ; △, water-sediment mixture ; ○, control. Dotted line and arrows indicate detectable limits of viral titres and less than the limits respectively.

3, 4, 5, and 6.

Since the loss of infectivities of IHNV and IPNV was noted only in untreated samples, further examination for actual virus inactivation by bacteria was accomplished by plaque reduction assay using 28 isolates from the water sample of the Moheji river at the end of incubation period. Culture filtrates of most of the strains reduced IHNV titre to more than 50% (Table 5). Among them, six strains showed

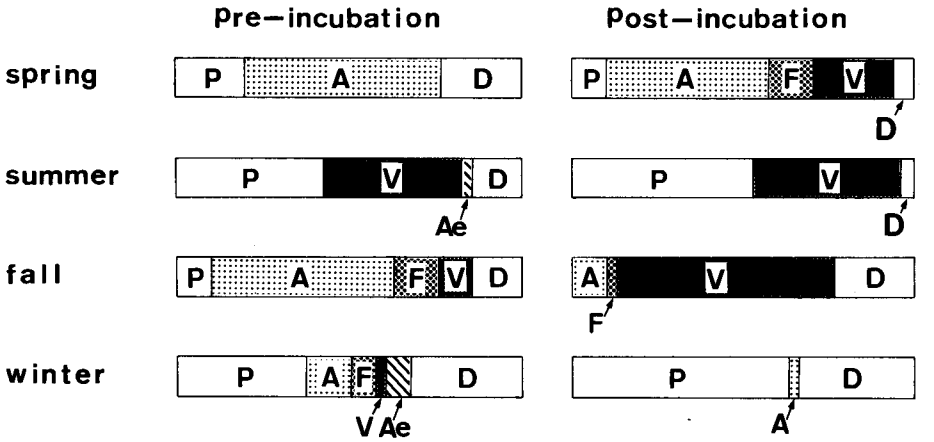

Fig. 3. Bacterial floras reducing the infectivity of IHNV in the water collected seasonally from 1984 to 1985 from the estuary of the Moheji river.
Symbols: P, *Pseudomonas*; A, *Achromobacter*; F, *Flavobacterium/Cytophaga*; V, *Vibrio*; Ae, *Aeromonas*; D, Dead and unidentified strains.

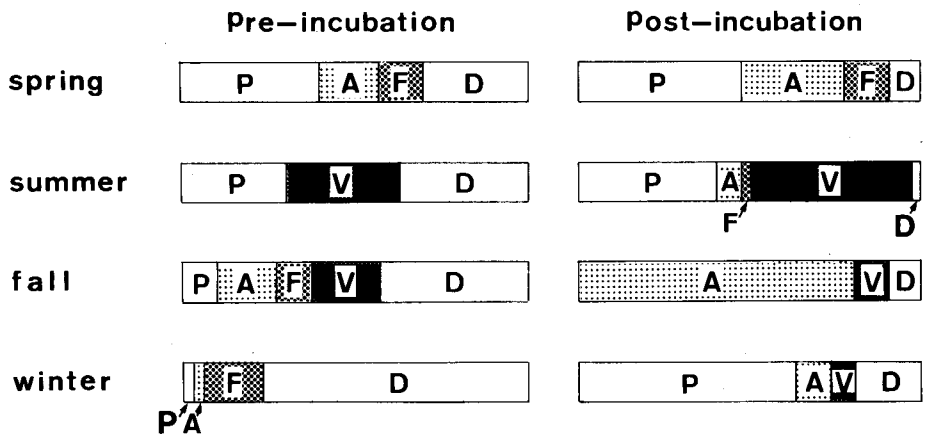

Fig. 4. Bacterial floras reducing the infectivity of IHNV in the sediment samples collected seasonally from 1984 to 1985 from the estuary of the Moheji river.
Symbols: P, *Pseudomonas*; A, *Achromobacter*; F, *Flavobacterium/Cytophaga*; V, *Vibrio*; D, Dead and unidentified strains.

plaque reduction more than 90%. The cultural pH after incubation ranged from 4.9 to 8.5, suggesting that there was no relationship between the cultural pH and antiviral activities to IHNV. Bacteria showed potent antiviral activity (>90% plaque reduction) belonged to *Achromobacter* sp. (46BBW-04, 46BBW-09, 46BBW-11, and 46BBW-25) and *Pseudomonas* sp. (46BBW-20 and 46BBW-22).

Using two representative strains (46BBW-09 and 46BBW-20) from each *Achromobacter* sp. and *Pseudomonas* sp., inactivation of IHNV by pure bacterial cultures in 50% ASW was investigated. Results obtained are illustrated in Fig. 7. Rapid inactivation of IHNV in the pure cultures was observed within 3 days of

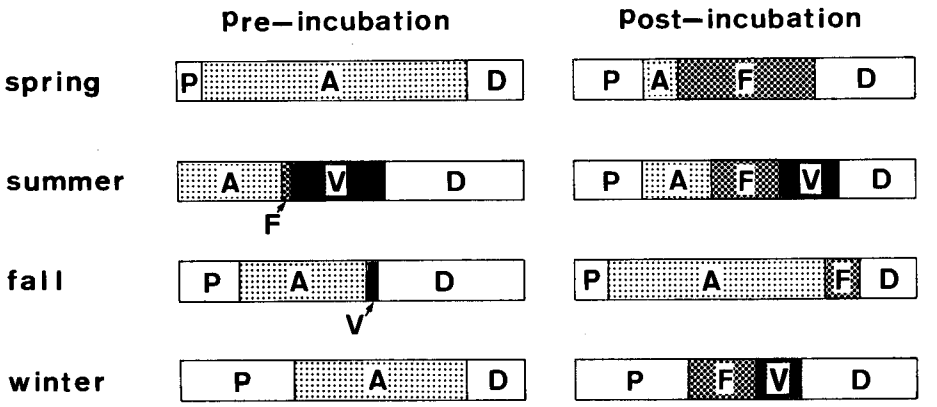

Fig. 5. Bacterial floras reducing the infectivity of IHNV in the water collected seasonally from 1984 to 1985 from the Nanaehama coast.
Symbols: P. *Pseudomonas*; A, *Achromobacter*; F, *Flavobacterium/Cytophaga*; V, *Vibrio*; D, Dead and unidentified strains.

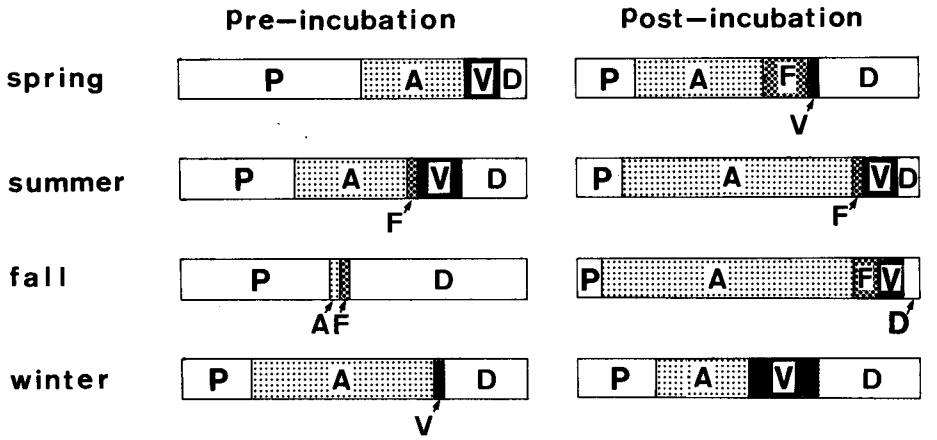

Fig. 6. Bacterial floras reducing the infectivity of IHNV in the sediment samples collected seasonally from 1984 to 1985 from the Nanaehama coast.
Symbols: P, *Pseudomonas*; A, *Achromobacter*; F, *Flavobacterium/Cytophaga*; V, *Vibrio*; D, Dead and unidentified strains.

incubation, following bacterial growth. This rapid inactivation did not occur continuously and the reduction rate of infectivity was moderate at 3 days after the bacterial growth reached the stationary phase.

Discussion

It has been reported that viruses are inactivated by chemical factors including halogens (Desautels and Mackelvie, 1975), formalin (Mackelvie and Desautels, 1975), reducing agents (Salo and Cliver, 1978), salinity (Lo et al., 1976; Pietsch et al., 1977), and ammonia (Ward and Ashley, 1977), and by physical factors such as

Table 5. Inactivation of IHNV by isolates from the brackish water sample at final incubation period.

Strain	pH[a]	Average of PFU on 2 wells	Plaque reduction[b]
46BBW-01	7.58	40	79.9%
46BBW-02	4.97	48	75.9%
46BBW-03	4.98	49	75.3%
46BBW-04	7.96	4	98.0%
46BBW-05	7.36	67	66.3%
46BBW-06	7.41	54	72.9%
46BBW-07	5.13	52	73.9%
46BBW-08	8.09	26	86.9%
46BBW-09	8.33	19	90.5%
46BBW-10	5.01	55	72.4%
46BBW-11	8.28	20	89.9%
46BBW-12	8.72	32	83.9%
46BBW-13	8.35	43	78.4%
46BBW-14	4.95	129	35.2%
46BBW-15	4.91	254	0.0%
46BBW-16	8.34	31	84.4%
46BBW-17	7.51	101	49.2%
46BBW-18	7.73	140	29.6%
46BBW-19	6.37	234	0.0%
46BBW-20	8.15	8	96.0%
46BBW-22	8.24	8	96.0%
46BBW-23	6.64	117	41.2%
46BBW-24	5.07	105	47.2%
46BBW-25	8.07	20	89.9%
46BBW-26	8.46	66	66.8%
46BBW-27	8.51	39	80.4%
46BBW-28	7.54	140	29.6%
46BBW-30	8.48	59	70.4%
Control (MCYG broth)		199	0.0%

[a]: cultural pHs of the test strains after incubated for 2 days;
[b]: plaque reduction rate was figured out by following formura:
$$\frac{\text{PFU of control} - \text{PFU of culture}}{\text{PFU of control}} \times 100.$$

temperature (Amend, 1970; Gosting and Gould, 1981; Lo et al., 1976; Mackelvie and Desautels, 1975; Yates et al., 1985) and ultraviolet light (Pietsch et al., 1977). Moreover, biological factors such as enzymes (Cliver and Herrmann, 1972), extracts from marine algae (Richards et al., 1978), an extracellular agents from bacteria (Toranzo et al., 1982, 1983a) have been shown to inactivate viruses.

In the present study, we collected brackish water from an estuary and seawater from a coastal environment to study the seasonal effects of water chemistry and

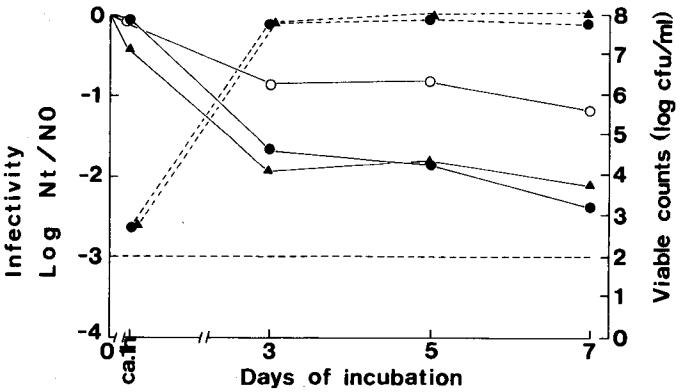

Fig. 7. Inactivation of IHNV by pure bacterial cultures of 46BBW-09 and 46BBW-20 in 50% artificial seawater at 15°C.
Symbols: —●—, virus inactivation by 46BBW-09; —▲—, virus inactivation by 46BBW-20; —○—, virus inactivation in sterile 50% artificial seawater; ···●···, viable counts of 46 BBW-09; ···▲···, viable counts of 46BBW-20. Dotted line indicates detectable limit of viral titre.

bacterial levels on the survival rate of the fish viruses, IHNV and IPNV. Our results showed that untreated water from estuarine and marine environments markedly reduced the infectivity of IHNV, irrespective of salinity concentration. IPNV was also inactivated in untreated brackish and sea water, but not in water sterilized by filtration and autoclaving. Similar results were obtained in each season, suggesting that seasonal variations in water quality do not have a direct effect on infectivities of the viruses.

It has been reported that estuarine sediment prolongs enteric virus survival (Labelle and Gerba, 1979; Labelle et al., 1980; Smith et al., 1978). In this study, we found that infectivities of IHNV and IPNV decreased in water containing sediment. The fact that these results differ from Smith et al. (1978) might be due to several factors: Smith et al. used 10% sediment compared with 1% used in this study; viruses used were different from enteroviruses; and the elution procedure for extracting viruses from sediment was not used in this study, though some investigators using various procedures successfully demonstrated viral release from sediment or sludge to which the virus had adsorbed (Flora et al., 1975; Gerba and Schaiberger, 1975; Wellings et al., 1976). Similar results have been obtained using a membrane dialysis chamber (Labelle and Gerba, 1980; O'brien and Newman, 1977). Research suggests that the protective effect of sediment on enteroviruses is attributed to adsorption and it is likedly to be directly affected by pH and salinity (Gerba and Schaiberger, 1975; Goyal and Gerba, 1979; Labelle and Gerba, 1979). In light of these findings, the fact that IHNV was inactivated by untreated samples or water containing sediment within 1 hour after suspension would be probably ascribable to physical factor such a virus adsorption to particles or sediment present in the water samples. Recently, Ward and Winston (1985) have exposed deficiencies and proposed improvements for virus inactivation studies. However, in our earlier research, culture filtrates of *Pseudomonas* isolates from the raceway water of salmonid hatchery reduced IHNV titre to less than detectable limits within 3-day

incubation period, but its autoclaved filtrates did not (Yoshimizu et al., 1986). In this report, we further demonstrated that culture filtrates of *Pseudomonas* and *Achromobacter* strains isolated from brackish water sample which IHNV inactivation was observed indeed inactivated the virus based on plaque reduction. Likewise, apparent IHNV inactivation in pure bacterial cultures supports the results obtained with environmental water. Furthermore, there are numerous other papers regarding virus inactivation in the aquatic environment that are concerned with microorganisms or extracellular substances from the microbes existent in the environment (Fujioka et al., 1980; Labelle et al., 1980; Magnusson et al., 1967; Matossian and Garabedian, 1967; Toranzo et al., 1982, 1983b). Thus, inactivation of fish pathogenic viruses in the aquatic environment appears to be due to bacterial products in addition to physical factors. The antiviral agents produced in the bacterial cultures will be described elsewhere.

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