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PREFACE

The United States and Japanese counterpart panels on aquaculture were formed in 1969 under the United States-Japan Cooperative Program in Natural Resources (UJNR). The panels currently include specialists drawn from the federal departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture which could be of benefit to both countries.

The UJNR was started by a proposal made during the Third Cabinet-Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, currents subjects included in the program are desalination of seawater, toxic microorganism, air pollution, water pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development, and utilization.

Accomplishments include: Increased communications and cooperation among technical specialists; exchanges of information, data and research findings; some 30 missions involving over 300 scientists and engineers; seven meetings of the Conference, a policy coordinative body; administration staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects on international relations.

This Proceedings represents the papers given at the Third Joint Meeting of U. S.-Japan Aquaculture Panel at Tokyo, Japan, October 15-16, 1974. Of great concern in aquaculture is the role diseases play. The following papers express this concern by relating the types of diseases found in aquaculture, methods of diagnois, and knowledge related to their limited control.

Lastly we wish to express our heartly thanks and appreciation to all those concerned with the organization of the Symposium. In particular, we are most grateful to the members of the Director Conference of Japanese National Fisherise Research Laboratory, who have never failed to extend their most experienced help to us in connection with the publication of the Proceedings. Further, we wish particularly to acknowledge the invaluable assistance of Mr. Hiroshi Fukataki —Japan Sea Regional Fisheries Research Laboratory—, who took the trouble of compiling the Proceedings.

ATSUSHI FURUKAWA—Japan
WILLIAM N. SHAW—United States

CONTENTS

A SUMMARY REPORT OF PACIFIC COAST OYSTER MORTALITY INVESTIGATIONS 1965–1972

JOHN B. GLUDE[1]

1. BACKGROUND

Significant mortalities of the Pacific oyster, *Crassostrea gigas*, were recorded in Puget Sound and Willapa Bay, Washington and in Humboldt Bay, California, during the late 1950's and, with increasing severity, during the early 1960's when losses reached 35 to 50%. The cause of these mortalities was not known, but oystermen and state shellfish biologists observed that mortalities weremost severe among fast-growing two-year-old oysters and occurred principally during the summer. It was feared that heavier and more wide-spread mortalities such as those caused by *Minchinia nelsoni* and *M. costalis* which had killed 75 to 90% of the oysters in Delaware and Chesapeake Bays in the late 1950's (Haskin, et al.; Couch, et al., 1966) might be in store for the Pacific Coast. In response to this threat to the Pacific oyster industry the Bureau of Commercial Fisheries of the Department of Interior[2] conducted a six year study to find the cause of these oyster mortalities.

2. ORGANIZATION OF INVESTIGATIONS

The urgency of the Pacific Coast oyster mortality problem made it important to utilize all of the technical skills available within various research groups on the Pacific Coast, as well as the experience in oyster pathology which had been developed at the National Marine Fisheries Service[3] (NMFS) Laboratoryat Oxford, Maryland. The research was contracted to various groups under the guidance of a steering committee composed of representatives of each group, and the oyster industry. The NMFS Regional Director, Seattle, Washington was assigned administrative supervision and coordinator of the program.

Research contracts covered these general areas:

1. California Department of Fish and Game-Monitoring of Pacific oyster mortalities at experimental stations in Humboldt Bay, Tomales Bay, Drakes Estero, Elkhorn Slough, and Morro Bay; histological examination of oyster tissues in search of suspected pathogens; studies of phytoplankton to investigate possible correlation with oyster mortalities.

2. Fish Commission of Oregon—Monitoring of growth and mortality of oysters in experimental plots in Yaquina, Tillamook, and Coos Bays; preservation of oyster samples for histological examination by specialists at the University of Washington; hydrographic observations at each station.

3. Washington Department of Fisheries—Preparation of slides from sample oysters provided by state fisheries agencies, examination of tissues for microoganisms suspected as pathogens. Representative slides and evaluation reports were returned to the state agencies and sent to the National Marine Fisheries Service Laboratory at Oxford, Maryland.

5. National Marine Fisheries Service, Oxford, Maryland—Examination of fresh and preserved samples of oysters from experimental plantings in Washington, Oregon, and California to determine to presence of microorganism suspected as pathogens; comparative studies of oysters from Japan, Korea, Taiwan, and the East Coast of the United States.

6. Battelle Northwest—Investigation of various research techniques which might be applicable to the investigation of oyster mortalities including testing of various stains and histological techniques which had been used with other species.

At the end of the three-year period covered by these research contracts, a critical review of progress was conducted by consultants Dr. Daniel B. Quayle from the Fisheries Research Board of Canada, Nanaimo, B. C., and Dr. J. D. Andrews, Virginia Institute of Marine Science, Gloucester Point, Virginia. The

[1] Deputy Regional Director, NMFS, Seattle Washington

[2] Now National Marine Fisheries Service, NOAA, Department of Commerce

[3] Formerly Bureau of Commercial Fisheries

results of this evaluation were used as the basis for revising research contracts for the following three-year period.

After the NMFS investigation was terminated in 1972, several important projects continued with other funding. Washington Department of Fisheries continued research and development on oyster culture techniques which could be used by the industry to reduce the adverse effects of mortality. California Department of Fish and Game continued pathological studies and field experiments in Humboldt Bay. In addition, a new study of the possible relation of bacteria of the genus *Vibrio* to oyster mortalities was undertaken by the University of Washington College of Fisheries under Sea Grant funding. Results of these projects will be reported separately by the sponsoring agencies.

3. CHARACTERISTICS OF OYSTER MORTALITIES

Oyster mortalities on the Pacific coast were characterized by heavy losses during the second summer in certain bays or portions of bays and low mortalities in other areas. Biologists of the Washington Department of Fisheries had conducted an annual survey to determine the average mortality of oysters in various parts of the State beginning in 1956, and from the results of these surveys were able to characterize certain estuaries or portions of estuaries as high or low mortaity areas.

Observations by Fish Commission of Oregon and records of oyster companies in California indicated that mortality in Oregon bays was uniformly low, whereas in California there is a great variation in mortality among the major oystering areas.

3.1. High Mortality Areas

High mortality areas were generally turbid, highly nutritive with muddy bottom, warm in summer, usually located near the head or upper end of estuaries. Examples in Washington were the upper part of Eld, Totten, and Case Inlets, and the tributaries or river estuaries of Willapa Bay. During the period 1956 through 1966 average mortality of oysters during the second summer after planting had been 26.3% in Eld Inlet, 19.1% in Case Inlet, 13.4% in Southern Puget Sound generally, and 8.7% in the Willapa River Estuary.

High mortality areas in California included the major portion of Humboldt Bay, and to a lesser extent, Tomales Bay. Average losses of oysters during their second summer in Humboldt Bay from 1961 through 1964, ranged from 34.2% to 56.4%. In Tomales Bay mortality during the second summer ranged from 35.9% in 1967 to 11.1% in 1971. These mortality rates excluded loss from known causes such as silting, storms, and predators such as crabs, oyster drills, and in California, the bat sting ray *Holorhinus californicus*.

3.2. Low Mortality Areas

Low mortalities were characteristically found in bays or toward the mouth or lower end of estuaries, where the water was clear, less productive, and usually colder, although some low mortality bays were as warm as some high mortality bays. In some low mortality areas, such as Hood Canal, Oakland Bay, and Grays Harbor, oysters did not fatten well, but in others such as the main part of Willapa Bay, Northern Puget Sound, and part of Southern Puget Sound growth rate and fattening were satisfactory. In the two major natural setting areas in Washington, Hood Canal and the main part of Willapa Bay, mortality rateswere low. Typical mortalities during the second summer after planting in low mortality areas in Washington based on surveys from 1956 through 1966 were 7.7% in Northern Puget Sound, 6.6% in Grays Harbor, and 7.5% in Willapa Bay.

In the three major oyster-producing areas of Oregon: mortalities were extremely low, averaging less than 2%—Yaquina Bay, 4%—Tillamook Bay and 7%—Coos Bay. In California low mortality was observed at Morro Bay 2.0 to 2.6%, Elkhorn Slough—2.7%, Drakes Estero—4.0 to 7.7%, and in parts of Humboldt Bay.

3.3. Mortality VS. AGE

Seed Pacific oysters are usually planted in the early spring when they are six to nine months old, and two to ten millimeters in diameter. Many of small oysters are killed by silting and predators, but mortality from unknown causes remained low during the first summer even in high mortality areas. Average mortalities for the first summer, based on observations of commercial planting over 10 or 11 years, indicated the following levels: Northern Puget Sound—5.2%; Southern Puget Sound—5.7%; Grays Harbor—4.9%; Willapa Bay—4.3%; Case Inlet—4.4%; Eld Inlet—6.0%; Willapa River Estuary—4.6%. In high mortality areas, the first major mortality generally occurred during the second summer after planting, and reached 20 to 30%. During severe mortality years losses of oysters in their third or fourth summer reached 30 to more than 50% in high mortality areas.

Table 1 shows mortality during the summer of 1967 for oysters in their second, third, and fourth summers in three high mortality areas: Case Inlet, Eld Inlet, and Totten Inlet, and two low mortality areas: Quil-

Table 1. Seasonal mortality of oysters of various ages in high and low mortality areas in Washington, 1967[1]

Age	High mortality areas			Low mortality areas	
	Case Inlet	Eld Inlet	Totten Inlet	Quilcene Bay	Oakland Bay
Second summer	23.3	28.0	32.0	1.3	10.0
Third summer	57.3	34.7	33.3	7.3	12.7
Fourth summer	44.0	24.3	41.3	5.0	14.0

[1] From Washington Department of Fisheries, Seasonal Summary Report No. 3.

Table 2. Percent mortality of Pacific oysters from unknown causes in three high and two low mortality areas in Washington and average temperatures[1]

Years	High mortality areas			Low mortality areas	
	Case inlet	Eld inlet	Totten inlet	Quilcene Bay	Oakland Bay
1956-1966	19	26	13		
1967	23	28	32	2	10
1968	25	30	9	3	18
1969	39	32	18	6	8
1970	29	55	24	12	6
1971	63	27	21	18	3
1972	9	10	10		
1973	16				
Average temperatureMay-Dec and Standard deviation					
1965	13.99-2.99	13.31-2.90	14.50-3.20	13.53-3.67	14.25-3.84
1966	14.28-3.09	13.40-3.26	14.44-3.88	13.94-4.22	15.06-3.33
1967	14.59-3.91	14.01-3.14	14.29-3.87	13.65-4.42	15.06-4.70

[1] Based on annual surveys of mortality on commercial beds by Washington Department of Fisheries.

cene Bay and Oakland Bay, Washington.

3.4. Oyster Mortalities in Japan

The mass mortality of oysters *Crassostrea gigas* in Matsushima Bay, Miyagi Prefecture, Japan, has occurred annually in late summer since 1961 (Kan-no, et al., 1965 and Imai, et al., 1968). Observed mortality rates were zero percent in July, 23% in August, 16% in September, and 8% in October with a total mortality of nearly 50%. Pathological observations indicated that an inflammation of the digestive diverticulae occurs during and after spawning, namely from August to October, coincident with the period of mass mortality. Imai comments, however, that according to a previous study (Tamate, et al., 1965) the simple inflammation of the digestive diverticulae could not be regarded as pathological, but as the normal physiological involution due to the sexual maturation and spawning of the oyster.

The similarities between mortality of oysters in Matsushima Bay, Japan and those observed in high mortality areas in the State of Washington are striking. In both places, (1) mortalities occurred only among rapidly growing oysters, (2) affected oysters were characterized by a rapid over-maturation of the gonads, (3) mortality occurred during the summer spawning period, and (4) no specific pathogen was related to the mortalities although a condition of degenerative necrosis of the digestive diverticulum was observed in parallel with mortalities. The major difference was the complete spawning of oysters in, Matsushima Bay and partial or lack of spwwning of oysters in Case, Eld and Totten Inlets in the State of Washington, and Humboldt Bay in California.

4. INVESTIGATIONS IN WASHINGTON

Washington Department of Fisheries had begun small scale investigations of causes of oyster mortality as early as 1956 and had conducted an annual survey to estimate oyster mortality in commercial plantings in various parts of the State and their efforts were expanded from 1965 to 1972 by Federal contracts. (Table 2 and Figure 1). Although certain areas were

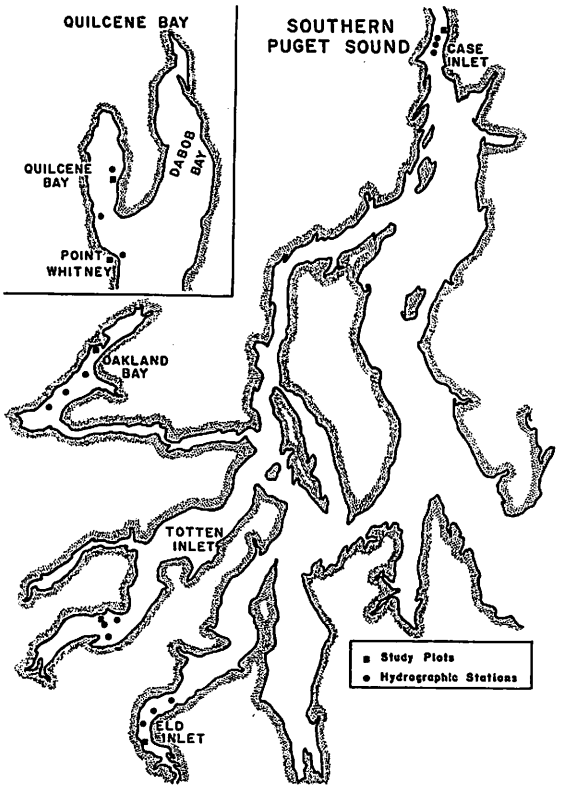


Figure 1. Location of experimental stations in Southern Puget Sound and Quilcene Bay, Washington.

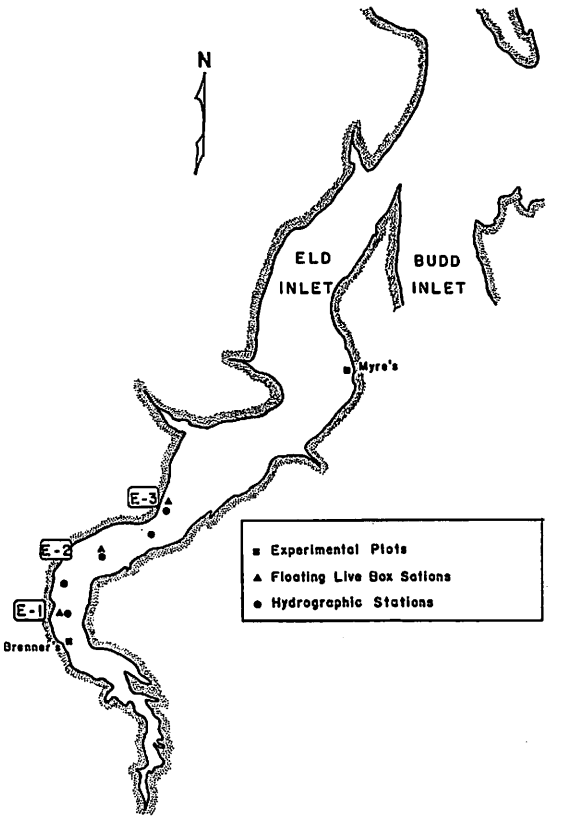


Figure 2. Location of experimental plots, floating live box stations and hydrographic stations in Eld Inlet in Southern Puget Sound, near Olympia, Washington.

characterized as high mortality or low mortality, rates varied from year to year. For example, mortality dropped to 9% in Totten Inlet, characteristically a high mortality area, in 1968. In contrast, mortalities of 18% occurred in Oakland Bay in 1968 and Quilcene Bay in 1971 even though these bays were characterized as low mortality areas.

4.1. Live Box Studies

Observations indicated a significant difference in oyster mortality at three locations within Eld Inlet (Figure 2). Near the head of the inlet, Station E-1, mortalities were high; at Station E-2, 1.25 miles towards the mouth, mortalities were moderate; and 1.25 miles further down by at Station E-3 mortalities were low. This situation provided an ideal opportunity for a comparative study.

Floating lives boxes were anchored at each station, and stocked with oysters approaching their second or third summer from a common source. The live box study was conducted during the summer of 1967, and repeated during the summers of 1968 and 1969. Observations included mortality, growth, condition index, gonadal development, histopathological evaluation offissue samples, temperature, phytoplankton (chlorophyll).

Seasonal cumulative mortality for oysters in the three locations were as follows:

Table 3. Floating livebox experiments—Eld Inlet, Wash. Seasonal cumulative mortality 1967–1969

Year	Station E-1	Station E-2	Station E-3
1967	40.7	6.7	3.3
1968	36.0	36.0	14.0
1969	35.9	21.0	13.5

Tables 4, 5, and 6 list periodic observations of mortality, temperature, chlorophyll, condition index, and average volume for 1967, 1968, and 1969 at locations E-1, E-2, and E-3 in Eld Inlet, wahington. Table 7 lists observations of gonadal development and sex ratio at the three locations for 1968, and table 8 summarizes the results of histopathological examinations of tissue samples from the three locations for 1968.

Table 6 indicates the relation between time of

Table 4. Floating livebox experiment-Eld Inlet, Washington 1967–1969. Monthly values of parameters at locations E-1, E-2, and E-3, 1967

Month	Mortality rate			Temperature			Chlorophyll "A"			Condition index			Average volume		
	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3
May								1.8							
June	6.0	0.7	0.0	17.1	16.3	14.3	16.1	13.9	5.7						
June	1.3	0.6	0.7	15.5	15.1	14.0		2.4							
June	1.8	0.9	0.0				5.7	5.0	0.8						
July	0.6	0.6	0.0	17.6	17.5	17.1	8.5	2.9	2.1						
July	3.0	1.8	0.5					1.3							
Aug.	3.6	0.6	0.6	20.3	18.3	16.7	5.8	2.9	82.1[1]						
Aug.	2.8	0.0	1.0	21.6	21.2	21.8	7.3	4.5	0.0						
								14.8							
Sept.	12.7	0.0	0.0	18.3	17.3	17.2	5.4	11.4	8.9						
Sept.	5.3	0.0	0.0												
Oct.	1.1	0.0	0.2												
Nov.															
Dec.	0.2	0.2	0.0							12.8	15.4	16.3	100.0	95.0	88.5

[1] Probably red tide bloom.

Table 5. Floating livebox experiments—Eld Inlet, Washington 1967–1969. Monthlyvalues of parameters at locations E-1, E-2, and E-3, 1968

Month	Mortality rate			Condition index		
	E-1	E-2	E-3	E-1	E-2	E-3
June	0	0	0	11.6	11.6	11.6
July	3.2	1.3	1.3	14.5	14.2	12.2
July	9.4	5.7	1.9	15.3	13.4	13.6
Aug.	10.7	11.8	4.3	14.3	13.3	13.1
Aug.	5.4	10.7	3.2	14.4	13.3	14.5
Sept.	14.5	9.1	3.3	12.5	12.9	13.3
Oct.	2.8	9.4	3.6	13.3	10.1	11.8
Nov.	4.1	4.4	2.2	10.2	10.2	11.4

Table 6. Floating livebox experiments—Eld Inlet, Washington 1967–1969. Monthly values of parameters at locations E-1, E-2, and E-3, 1969

Month	Mortality rate			Temperature			Condition index			Volume		
	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3
April				9.8	9.7	9.5	6.2	6.2	6.2	33.0	33.0	33.0
May	0.8	0.9	0.9	16.5	16.9	16.1	13.5	13.6	13.1	39.0	44.0	49.5
June				16.6	15.1	14.1						
June	0.3	0.5	0.3	14.9	14.2	14.4	16.4	16.3	17.8	51.5	48.3	40.3
July				17.2	15.7	15.8						
July	8.6	2.0	1.0	19.0	17.4	17.8	17.6	16.8	16.6	45.3[2]	47.0	49.8
Aug.				17.3	16.8	16.6						
Aug.	10.7	7.7	4.6	16.4	15.8	15.6	12.8[1]	10.4[1]	17.7	52.5	45.0	45.0[2]
Sept.				15.6	15.3	15.0						
Sept.	12.1	8.1	6.4	15.6	15.2	14.7	13.5	16.1	16.9	52.1	35.0[2]	38.5[2]
Oct.	5.4	3.5	1.4	12.1	12.2	12.8	13.9	19.3	23.7	52.9	51.5	59.5
Nov.				10.0	10.1	10.3						

[1] Decrease because of spawning. [2] Decrease because of mortality of larger oysters.

spawning, as indicated by the decrease in condition index between July 22 and August 18 for floats E-1 and E-2, and the increase in mortality rates at these locations.

It is interesting to note from Table 7 that the percentage of males in samples removed for evaluation of gonad development was 28.2 in float E-1, 32.8 in float E-2, and 39.3 at float E-3, even though all of the oysters came from the same stock. These data may indicate that the mortality was selective toward males.

Table 8 summarizes the occurrence of the ciliate *Ancistrocoma*, the parasitic copepod *Mytilicola* and the conditions termed bacterial foci and tissue necrosis. Neither *Ancistrocoma* nor *Mytilicola* are recognized as causes of mass mortalities. Incidence of bacterial foci was low and did not correlate with the observed mortalities. Tissue necrosis, however, occurred in seventeen percent of the oysters sampled from the high mortality area, E-1, 13% from the intermediate mortality area, E-2, and 4% from the low mortality area, E-3.

Table 7. Floating livebox experiments, Eld Inlet, Washington 1967–1969. Gonadal development May–October 1968 and sex ratio of oysters in samples from locations E-1, E-2, and E-3

Date	Gonadal development				Sex of sample oysters	
	Development	Mature	Spawn	Resorb	Male	Female
Location—E-1						
6-21	97	0	0	3	13	16
6-23	24	59	14	4	8	17
7-8	0	30	63	7	6	24
7-22	0	67	26	7	11	17
8-4	3	67	0	30	10	20
8-20	0	50	3	47	5	24
9-4	0	55	0	45	5	24
9-15	3	50	0	47	10	19
9-16	0	36	0	64	4	22
Total	127	414	106	254	72 (28.2%)	183
Location—E-2						
6-21	100	0	0	0	9	16
6-23	10	70	20	0	14	16
7-8	4	24	62	10	8	20
7-22	3	53	30	13	10	19
8-4	4	69	7	21	11	18
8-20	0	57	7	37	9	19
9-4	3	57	0	40	9	21
9-15	0	67	0	33	7	23
9-16	0	40	0	60	8	22
Total	124	437	126	214	85 (32.8%)	174
Location—E-3						
6-21	93	7	0	0	15	14
6-23	0	67	23	10	7	22
7-8	7	38	52	4	13	15
7-22	0	59	31	10	8	21
8-4	0	63	20	17	10	19
8-20	0	73	17	10	13	16
9-4	3	90	0	7	12	18
9-15	0	67	3	30	12	17
9-16	0	53	0	47	13	17
Total	103	517	146	135	103 (39.3%)	159

Table 8. Floating livebox experiments, Eld Inlet, Washington 1967–1969. Summary of histo-pathological examination of 64 tissue samples from locations E-1 and E-2 and 71 from E-3, 1968.

Date	Ancistrocoma			Mytilicola			Bacterial foci			Tissue necrosis		
	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3	E-1	E-2	E-3
July 15	4	0	3	3	3	0	0	1	0	0	0	0
July 31	2	5	2	2	0	0	2	0	1	2	3	0
Aug. 14	2	1	3	2	0	0	1	1	1	0	0	0
Aug. 28	4	0	1	0	0	1	0	0	0	6	1	1
Sept. 26	3	0	2	2	1	0	0	1	1	1	2	0
Oct. 24	1	1	2	2	2	2	0	0	0	2	2	0
Total	16	7	13	11	6	3	3	3	3	11	8	3
Percent	25	11	18	17	9	4	5	5	4	17	13	4

(Seasonal Mortality: E-1=36%; E-2=36%; E-3=14%)

Table 9. Oyster transplanting experiment, Eld Inlet, Washington 1967 cumulative mortality of control and transplanted oysters at Brenner's (a high mortality area) and Myre's (a low mortality area)

	Transplanted stocks					
Date of cross-transplant	Tray No.	Pre-transplant[1] mortality (at Brenner's)	Post-transplant[2] mortality (at Myre's)	Tray No.	Pre-transplant[1] mortality (at Myre's)	Post-transplant[2] mortality (at Brenner's)
May 30	1	0.0	0.0	16	0.0	27.5
June 9	2	0.0	0.0	17	0.0	30.0
June 19	3	3.3	1.7	18	1.6	36.7
June 29	4	6.7	3.3	19	1.7	25.0
July 9	5	5.0	6.7	20	6.6	36.7
July 19	6	5.0	11.7	21	5.0	23.3
July 29	7	1.0	6.7	22	0.0	13.3
Aug. 8	8	11.6	11.7	23	0.0	13.3
Aug. 18	9	8.3	6.7	24	3.3	16.7
Aug. 28	10	18.4	13.3	25	8.4	8.3
Sept. 7	11	21.6	6.7	26	10.0	3.3
Sept. 17	12	15.0	13.3	27	6.7	11.6
Sept. 27	13	36.7	3.3	28	5.0	0.0
Oct. 7	14	28.4	3.3	29	3.4	13.3
Oct. 17	15	20.0	1.7	30	6.7	0.0

[1] Pre-transplant mortality is that from April 19 to the cross-transplant date.
[2] Post-transplant mortality is that from the cross-transplant date to November 29.

Note:

Control stocks			
Myre's		Brenner's	
Tray No.	Total mortality[3]	Tray No.	Total mortality
35	6.7	31	30.0
36	1.7	32	26.7
37	8.3	33	30.0
38	6.7	34	31.7

[3] Total mortality is that from April to November 29.

Examination of 32 live oysters taken from commercial beds in Eld Inlet on August 8, 1928 indicated that 12 (37.5%) showed tissue necrosis without any observed bacteria. September and October samples at the same location indicated that oysters with unexplained necrosis had decreased to low levels (8.3% and 6.2% respectively) indicating a positive correlation with observed mortality. The relation between tissue necrosis in Washington oysters and inflammation of the digestive diverticula of oysters from Matsushima Bay Japan, reported by Imai, et al, 1968 is not clear. Illustrations in the report by Imai, et al, appear more like the condition in the United States described as bacterial foci.

4.2. Transplanting Experiments

A transplanting study was conducted in Eld Inlet using a known high mortality area (Brenners) and a low mortality area (Myres), figure 2. Nineteen compartmentalized trays, each initially containing a total of 60, 2+ age oysters (three replicate groups of 20 oysters per tray) were placed at Brenners and at Myres oyster beds. Four trays (12 replicates) at each station were designated as controls. Beginning on May 30, 1967 and continuing at 10 day intervals until October 17, one tray from each location was cross-transplanted to the other station. Every ten days the control oysters and all transplanted oysters were examined to determine mortality rate. No oysters were transplanted more than once during the study.

Table 9 shows the mortality rate of transplanted stocks and control stocks for both Brenners and Myres Stations. Mortality of control oysters at Brenners (29.6%) was significantly higher than mortality of control oysters at Myres (5.9%). Oysters transplanted from the high mortality area (Brenners) to the low mortality area (Myres) before June 30 did not differ significantly in mortality from control oysters at Myres. Lots moved from Brenners to Myres after June 30 had significantly higher mortality rate than the control oysters at Myres. This indicated that causative condition was present at Brenners at the beginning of July and that once exposed to this condition, oysters moved from that area were still subject to its effects.

Lots of oysters transplanted from the low mortality area (Myres) to the high mortality area (Brenners) from May 30 through July 19 had post-transplant mortality rates equal to or slightly less than the control stock at Brenners. Lots transplanted from Myres to Brenners after July 19 had post-transplant mortality rates intermediate between those of control oysters at Brenners and those at Myres. At least five of the fifteen groups transplanted to the high mortality area began to show mortality immediately after transplant, which suggests that no specific pathogen was responsible for the mortalities.

Mortality data for the twelve control replicates at Brenners were tested to find if an unidentified contagious disease was responsible for the mortalities. This was done by determining if oyster deaths in each replicate were independent of what happened to other oysters in that compartment. The results of chi-square analysis of these data indicated no evidence of a contagious disease.

4.3. Seed Source Study

Seed oysters used by the Pacific oyster industry of the U.S. West coast come from a variety of locations. A major source is Matsushina Bay in Japan but within this area there are a number of localties where seed oysters are collected for export. Another major source of seed oysters is Pendrell Sound, British Columbia, Canada where surface waters become warm enough to provide a satisfactory environment for reproduction of Pacific oysters, The two major seed sources in the United States are Dabob Bay in Hood Canal, and Willapa Bay Washington.

Since oyster seed from various sources had been used in experimental plantings in Washington, Oregon, and California to monitor survival, it seemed important to determine the effect of seed source on mortality rates, especially during the second summer after planting. The plan of the seed source experiment was to obtain seed from five major seed producing areas and plant it in two high mortality bays, one in Washington and one in California. Locations chosen for planting experiments were North Bay in Case Inlet, Washington, and Humboldt Bay, California. The experiment was begun in May, 1968, and continued through 1971.

Seed oysters were obtained from two locations in Japan to test the possibility that genetic resistance to the mass mortality which occurs in mid summer in Matsushima Bay might have occurred. Seed oysters packed at Hojima in Matsushima Bay were from parent oysters which had survived the mass mortalities and therefore might have developed some genetic resistance. Oyster seed packed at Mangoku-ura originated in an area of low adult mortality where genetic resistance would not be expected because the parent oysters presumably had not been exposed to the causative agent of the Matsushima Bay mortalities.

Dabob Bay in Washington and Pendell Sound in British Columbia had adult populations which had been isolated from recent importations of Japanese seed and neither area was subject to the mass mortal-

ities. Therefore, it seemed improbable that these oysters would have genetic resistance to the causative agent of mass mortalities in Washington State or California.

Willapa Bay in Washington State had received large quantities of imported Japanese seed each year and included areas where oyster reproduction was successful. Unexplained mass mortalities had occurred in parts of Willapa Bay, although with less regularity than in some parts of Puget Sound. Seed oysters from Willapa Bay might have been the progeny of adults which had survived mass mortalities and therefore might have been expected to have developed some genetic resistance.

Ten standard cases, or the equivalent in shell strings, of 1967 catch oyster seed were obtained from Mangoku-ura and Hojima packing sites in Japan; Dabob Bay, and Willapa Bay in Washington; and Pendrell Sound, Canada. Half of the seed from each of the five groups was planted in Humboldt Bay, California. The other half of the seed was planted on the North Bay State Oyster Reserve in Case Inlet, Washington at about the +1 foot tide level during May 1968. About 300 pieces of shell with attached spat from each group were placed in trays made of one inch mesh hardware cloth laid directly on the substrate to estimate mortality and growth during the first six months of the experiment. The rest of the seed was directly upon the substrate in five parallel plantings.

Mortality during the first six months ranged from 46.1% to 75% and seed which started with the highest counts of spat per shell suffered the greatest reduction in numbers (Table 16).

During the second summer, 1969, mortalities ranged from 2.4% for Willapa Bay seed to 31.9% for Hojima seed. At the end of the experiment Dabob Bay seed which had started the highest count per shell was still the highest, Pendrell Sound second, and seed from the other three sources were somewhat lower.

A comparsion of results of the Washigton and California portions of this experiment are included in part 6 of this report. No conclusions could be drawn from this experiment regarding genetic resistance or genetic superiority of any stock for culture in mass mortality areas due to variabilites in the initial spat size and spat density. Even though Hojima seed had higher mortality rates during the second summer, the final number of spat per shell was equal to or better than that of seed from Willapa Bay or Mangoku-ura.

4.4. Ceratium Theory

Blooms of the armored dinoflagellate *Ceratium fusus* occur periodically in Southern Puget Sound. Since these blooms usually occur during the hottest part of the summer at the peak of oyster mortality it was theorized that this phytoplankter or its waste products killed oysters. To test this theory Washington Department of Fisheries determined the occurrence of *C. fusus* from May to August 1967 in Case Inlet, Eld Inlet, Totten Inlet, Quilcene Bay, and Oakland Bay (Table 10).

Table 10. Occurrence of *Ceratium fusus* (cells per liter) from May to August 1967 in study areas, in Washington

Sample date	Area				
	Case Inlet	Eld Inlet	Oakland Bay	Quilcene Bay	Totten Inlet
May 18	0	0	0	0	0
June 2	0	22,000	—	—	—
June 13	0	0	0	6,600	3,000
June 26	0	0	—	—	—
July 6	—	3,000	—	—	—
July 12	0	2,000	0	3,000	6,000
Aug. 2	0	1,156,000	—	—	—
Aug. 15	0	336,000	—	—	—
Aug. 23	0	427,000	3,000	0	34,000

— Indicates no sample taken.

High levels of *C. fusus* occurred in Eld Inlet concurrent with high levels of oyster morality (28%, table 2) but in Case Inlet, where oysters suffered a high mortality (23%, Table 2), no *C. fusus* were found. In Totten Inlet relatively few *C. fusus* were found, but oyster mortality reached 32%. These observations ruled out *Ceratium fusus* as a direct cause of oyster mortaliity and the plankton sampling and enumeration project was discontinued.

Similar studies in Humboldt Bay, California are described in part 6 of this report.

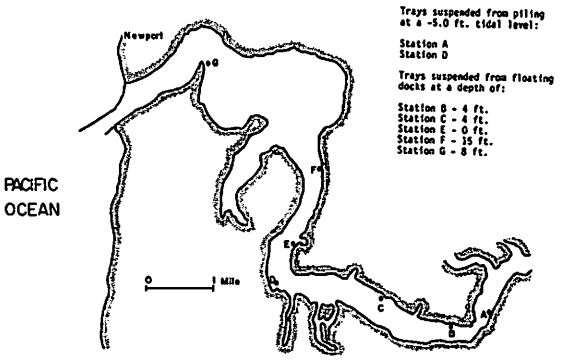
5. INVESTIGATIONS IN OREGON

The principal purpose of the studies in Oregon was to provide low-mortality control areas for comparison with the high mortality areas in Washington and California.

5.1. Mortality Studies

Experimental plantings of one to three year old oysters were made periodically in Yaquina Bay, Tillamook Bay, and Coos Bay. Seven subtidal tray stations were established in Yaquina Bay, Figure 3, and reduced to four stations at the end of two years due to similarity of mortality among stations. During the fifth year the number of stations in Yaquina Bay was reduced to three, and during the sixth year to two. Intitial populations of four hundred [Pacific oysters

were placed at each location and samples were taken monthly for histopathological studies by the University of Washington or the Federal Laboratory at Oxford, Maryland.



Fgure 3. Location of experimental stations in Yaquina Bay near Newport, Oregon.

Single intertidal stations were established within commercial oyster beds in Tillamook and Coos Bays. Each station initially contained 50 Pacific oysters, one to three years old, for the first study year, 100 oysters the second and third year, and 150 for the last three years. Experimental plantings were checked monthiy during the first four years and quarterly during the last two. Samples were removed each time for histopathological studies.

Mortality of oysters from unknown causes in experimental plantings in in Oregon was consistently low except in Coos Bay during the summer of 1968 when 19% of the oysters died. No similar mortality was observed in adjacent commercial plantings (Table 11).

Table 11. Percent mortality of Pacific oysters at experimental stations in Oregon 1966–1972

Date	Yaquina Bay 3–7 stations	Tillamook Bay 1 station	Coos Bay 1 station
7/66–6/67	1.8	4.0[2]	12.0[4]
7/67–3/68	1.5	2.4	7.3
4/68–3/69	1.1	4.6	27.1[5]
4/69–3/70	8.6[1]	2.1	6.9
4/70–3/71	2.0	7.3	6.7
4/71–3/72	1.8	[3]	4.0

[1] Half in 1 tray, upper bay, July—Sept 1969.
[2] Oct 66—Apr 1967.
[3] Unknown; most buried by storm.
[4] Jan—June 1967.
[5] 19.0% summer 1968—cause undetermined. No similar mortality in adjacent commerical plantings.

5.2. Other Observations

Experimental plantings of Pacific oysters in three

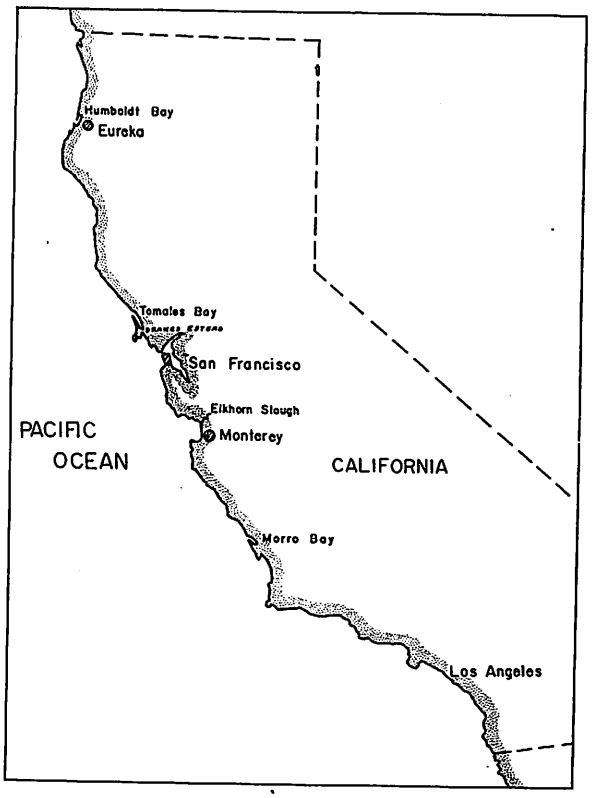


Figure 4. Location of oyster producing areas in California included in mortality investigation.

Oregon bays provided an opportunity for several associated studies. Shell growth of Pacific oysters in suspended trays in Yaquina Bay was measured during 1968 and 1969, at stations shown in Figure 4. During two years in suspended trays oysters at Station E became 41.5% larger than those at Station B and 28.4% larger than oysters at Station C. According to Gibson (1974) the higher salinities during the winter months and intense algal blooms during spring and summer months probably contributed to the rapid growth of oysters at Station E.

In addition Gibson determined salinity, temperature, dissolved oxygen, turbidity, and nutrients from water samples taken at stations in Yaquina Bay and tabulated condition index of Pacific oysters in Yaquina, Tillamook, and Coos Bays, June 1966 to March 1968.

5.3. Incidental Studies of Native Oysters and Mussels

The establishment of experimental plots in Oregon provided an opportunity for comparative studies of Pacific oysters, the native oyster *Ostrea lurida* and the blue or bay mussel *Mytilus edulis* and several species of clams which were included among the histological

samples sent to University of Washington or the Federal Laboratory at Oxford, Maryland for study. Studies of these samples resulted in discovery of a "neoplastic disease" of native oysters and bay mussels from Yaquina Bay (Jones and Sparks, 1969; Farley, 1969; and Farley and Sparks, 1969).

6. INVESTIGATIONS IN CALIFORNIA

Investigations of oyster mortalities in California were conducted under contract by the California Department of Fish and Game, from 1966 to 1972. Observations of the pattern of mortality of commercial and experimental Pacific oyster populations were made at all of the major oyster culture areas in California: Morro Bay Elkhorn Slough, Drakes Estero, Tomales Bay, and Humboldt Bay, as shown in Figure 4.

In each bay, experimental oyster populations were placed in 40″×24″×7″ rubberized wire baskets or trays held several inches above the oyster beds to minimize the effects of silting and predation. At least 10 live oysters of each year class and all gapers (moribund or dead oysters with soft parts intact) were removed from each experimental area each month. More frequent samples were taken during the high mortality period, May through September. During periods of heavy losses, gapers and live oysters were also collected from commercial oyster beds.

Samples were prepared for histopathological studies by removing a quarter inch cross section of tissue posterior to the palps and fixing this in Davidson's solution (Shaw and Battle, 1957.). The tissue was then dehydrated, cleared, infiltrated with paraplast and then blocked in paraplast. Sections of tissue were cut on a rotary microtome at 7 microns, cleared, rehydrated, and stained with hematoxylin and eosin. Special stains for connective tissue, spores, bacteria, etc. were occasionally employed.

Histopathological studies were conducted by Dr. Stanley Katkansky, pathologist of the California Department of Fish and Game and slides were sent to pathologists at the University of Washington and at the NMFS laboratory at Oxford, Maryland for further study.

Field studies provided an opportunity to collect other mollusks associated with oysters. Histological studies resulted in several published reports (Katkansky, Dahlstrom and Warner, 1969; Katkansky and Warner, 1969; Katkansky, Warner & Poole 1969).

6.1. Morro Bay

Two experimental stations were established in Morro Bay in November, 1966 (Figure 5). Four baskets were placed at each station and stocked with Pacific oysters from Japnese seed which had been planted at Drakes Estero during the spring of 1966. These two stations were monitored from January, 1976 through March 1968, at which time they were discontinued because of the low mortalities which were observed. Losses at the Baywood Park Station were 2.0% and at the Los Osos station 2.6% during this period.

Histological examination of oysters from Morro Bay revealed no organisms which could be considered potentially pathogenic. The endoparasitic copepod, *Mytilicola orientalis*, the most frequently observed

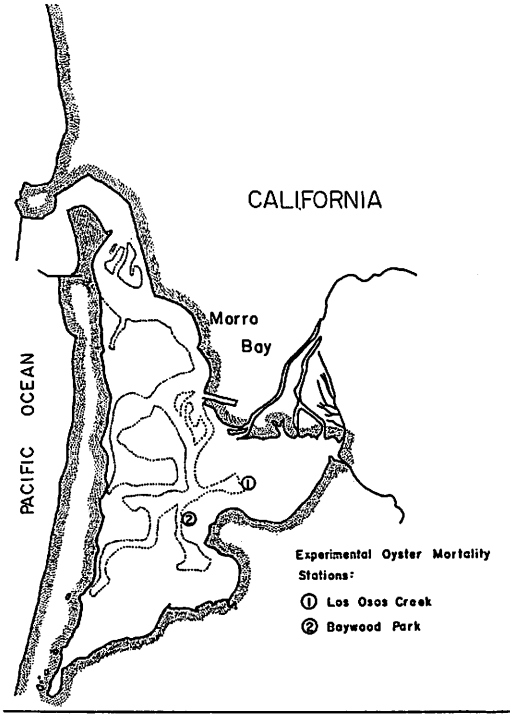


Figure 5. Location of experimental stations in Morro Bay, California.

Figure 6. Ancistrocomid ciliates in the digestive diverticulae of the Pacific oyster in California.

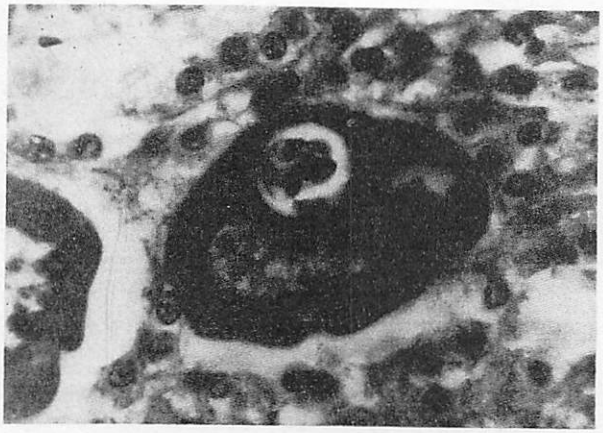


Figure 7. An unidentified parasite in the ova of the Pacific oyster in California.

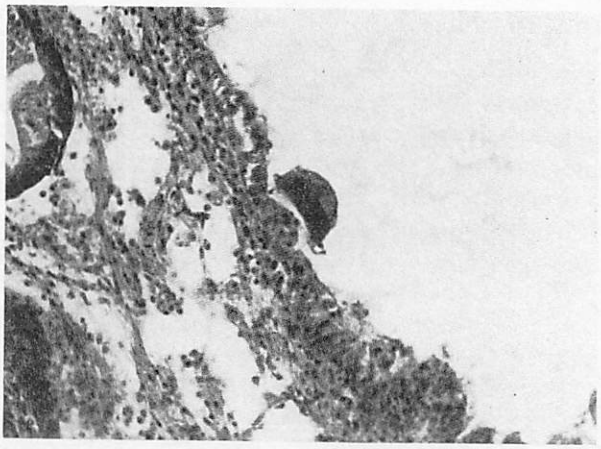


Figure 8. *Trichodina* like ciliate on a Pacific oyster in California.

parasite, was present in 25–40% of the oysters examined. Ciliates, *Ancistrocoma*, (Fig. 6) were observed in the digestive diverticulae of up to 12% of the Pacific oysters examined.

An unidentified ovarian parasite (Figure 7) was observed in female oysters but was not considered to have a detrimental effect on the oyster due to the low incidence of infection. (Becker and Pauley, 1968). It was also observed that infected ova were phagocytized by the oyster's leucocytes, indicating the possibility of the host ridding itself of at least some of the parasites. Although heavy infections might reduce the reproductive potential of the population this was not considered to be an important factor in California where commercially successful natural reproduction of Pacific oysters does not occur.

Peritrichous ciliates probably of the genus Trichodina (Figure 8) were noted on the gills and mantles of an occasional Pacific oyster but were not considered as pathogens.

Large numbers of encysted cestode plerocercoids, *Echeneibothrium* sp. (Figures 9 and 10) were found in rough-sided littleneck clams, *Protothaca laciniata* collected near the Los Osos creek station in Morro Bay (Katkansky and Warner, 1969).

Histological examination of approximately 350 oysters from this area showed two *O. edulis* and five *C. gigas* to have similar plerocercoids in their intestinal tract but in no instance were plerocercoids observed to be encysted in the tissues of the oysters. It was assumed that the larval cestodes entered by way of the oysters' normal feeding mechanisms, metamorphosed and would probably have been voided.

European flat oysters, *Ostrea edulis* from seed reared at the NMFS Biological laboratory at Milford, Connecticut had been planted experimentally in Morro Bay in 1963, 1964 and 1965 by the California Department of Fish and Game but survival and growth of these oysters was poor. In December 1965 a amples

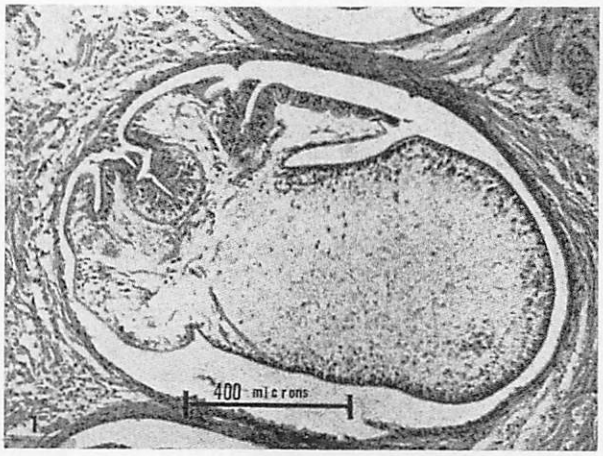


Figure 9. A single encysted *Echeneibothrium* sp. from the clam *Protothaca laciniata* in California.

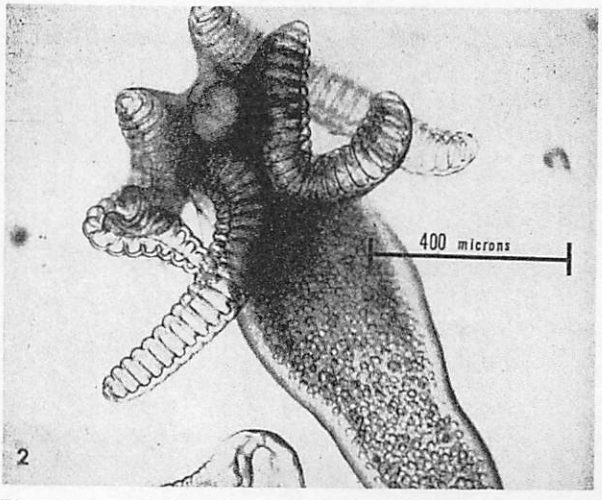


Figure 10. A living plerocercoid, *Echeneibothrium* sp. teased from a cyst in the tissues of a parasitized clam, *Protothaca laciniata* in California.

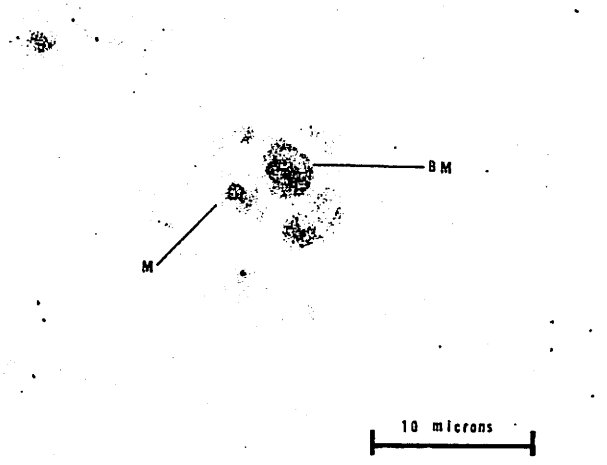


Figure 11. Microcells cytozoic within a leucocyte of a European oyster *Ostrea edulis* in California.

of live oysters was forwarded to the NMFS Biological Laboratory at Oxford, Maryland for study and an organism closely resembling one associated with Pacific oyster mortalities on the West coast of Canada was found (C. J. Sindermann, Pers. Comm.). This organism described as a "microcell" was two to three microns in diameter and contained a single nucleus of approximately 0.8 microns (Figure 11). Observations by Dr. Sindermann and his associates indicated that the organism was cytozoic within leucocytes or was free in the blood, and division appeared to be amitotic.

The California Department of Fish and Game pathologist, Stanley Katkansky, confirmed the presence of microcells in thin, watery or transparent oysters *O. edulis* from Morro Bay. Histologically, the infected oysters exhibited a generalized leucocytic infiltration which was partially evident in the area of the digestive diverticulae. The digestive diverticulae generally lacked the normal crypt structure, with the epithelial cells being reduced to a low cuboidal epithelium similar to the condition described in Pacific oysters by Pauley and Sparks, 1965. Microcells were observed in all gaping European oysters from Morro Bay and in approximately 30% of the live oysters (Katkansky, Dahlstrom and Warner, 1969).

Pacific oysters were held in close proximity of European oysters in Morro Bay; however, no microcell infections were observed in Pacific oysters. As a precaution however, the infected European oysters were removed from areas where Pacific oysters were being cultured because of Pacific oyster mortalities at Denman Island, British Columbia, Canada from what was reported as a "microcell disase."

6.2. Elkhorn Slough

During the 1930's Elkhorn Slough produced significant quantities of Pacific oysters but at the time of the initiation of this study only a few were being commercially harvested for local market.

One experimental station was established in Elkhorn Slough (Figure 12) in November 1966, and monitored through March 1968. Six baskets were placed on a redwood rack near the small commercial oyster bed and stocked with oysters from Japanese seed planted in Drakes Estero during the spring of 1966. This was the same stock of oysters used for plantings in Morro Bay. Total mortalities during 1967 were 2.7% and only 0.6% for January to March 1968 when this experiment was terminated.

Although histological examination of sample oysters from the Elkhorn Slough experiment revealed no organisms which might be considered pathogenic, parasites similar to those found in oysters from Morro Bay were observed. Of the 350 Pacific oysters examined, *Mytilicola orientalis* was found in 5 to 15%; Ancistrocomid ciliates in the digestive diverticulae of up to 15% and ovarian parasites in 12% to 40% of the oysters examined. Plerocercoids of a cestode *Echeneibothrium* (sp.) were observed in the intestinal tract of

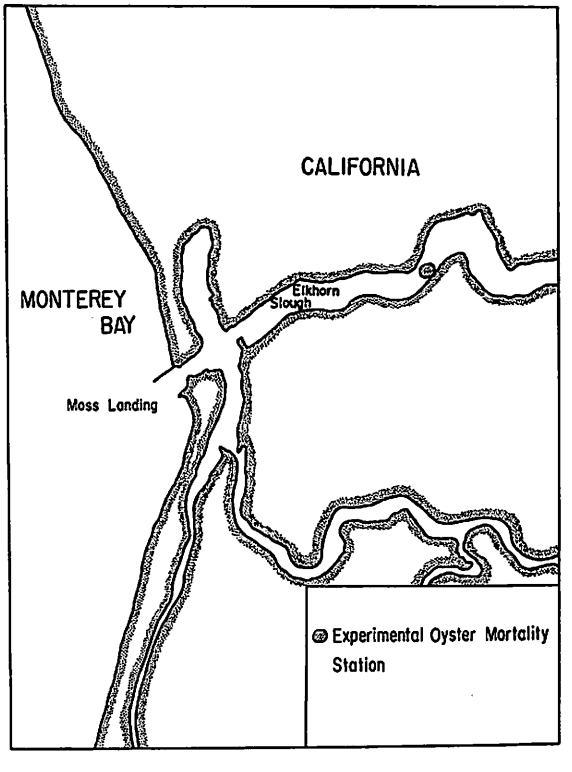


Figure 12. Location of experimental station in Elkhorn Slough near Moss Landing, Cellfornia.

four Pacific oysters.

In March 1966, 298 European oysters (*O. edulis*) from a 1965 planting in Tomales Bay were transferred to Elkhorn Slough. Mortality rates of these oysters reached 100% by March 1967. The gross and histological appearance of these oysters was identical to those at Morro Bay. All gapers and approximately 58% of the live oysters examined were infected by microcells. These European oysters were held in close proximity to Pacific oysters but no cross infection was observed.

6.3. Drakes Estero

Two experimental stations were established in Drakes Estero (Figure 13) in December 1966. Each station consisted of four wire baskets supported above the bottom on a redwood rack and stocked initially with Pacific oysters of the 1966 seed planting in Drakes Estero and later with oysters of the 1967 year class. Cumulative mortality rates for these experimental populations are given in Table 12.

During the period of observation in Drakes Estero commercial oyster growing by the traditional ground culture methods was being abandoned for rack culture (Figure 14). Beginning in January 1970 the two experimental stations in Drakes Estero were discontinued and baskets containing oysters of the 1967 and 1969 plantings were hung from the racks to more closely simulate the conditions of commercial culture. Losses among these two plantings during 1970 were 4.0% and 7.7% respectively. Because of the low mortalities among experimental and commercial stocks most of which were reported to have been the result of predation by rock crabs (*Cancer productus*) this experiment was terminated in January 1971.

Ironically, during the latter part of August and early September 1971 heavy losses were reported among the commercial rack-cultured oysters in Drakes Estero. It was estimated that nearly 62% of these oysters died within a short period of time. Histological examination of limited samples indicated no obvious pathological condition. It was the contention of the oyster culturist in the area that this mortality was the result of unusually high air tempera tures during a period of low tides when the oysters were exposed.

Throughout the period of study no potentially pathogenic organisms were observed among the samples taken for histological examination and the parasites observed were similar to those found in Morro Bay and Elkhorn Slough. The unidentified ovarian

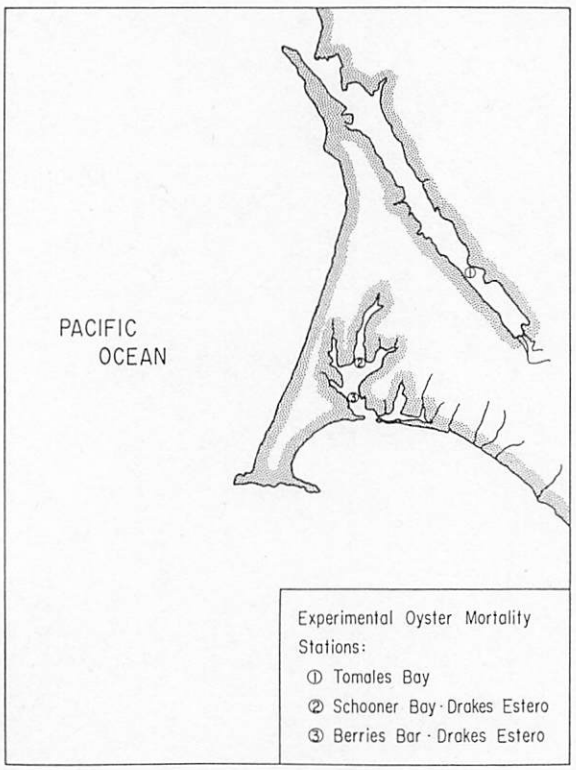


Figure 13. Location of experimental stations in Drakes Estero and Tomales Bay, California.

Table 12. Percent mortality of 1966 and 1967 year class Pacific oysters at two stations in Drakes Estero, California 1967–1969

Year	Schooner Bay station		Berries Bar station	
	1966 Planting	1967 Planting	1966 Planting	1967 Planting
1967	14.2		7.0	
1968	10.4	6.9	3.8	6.0
1969	6.0	12.1	2.0	6.1

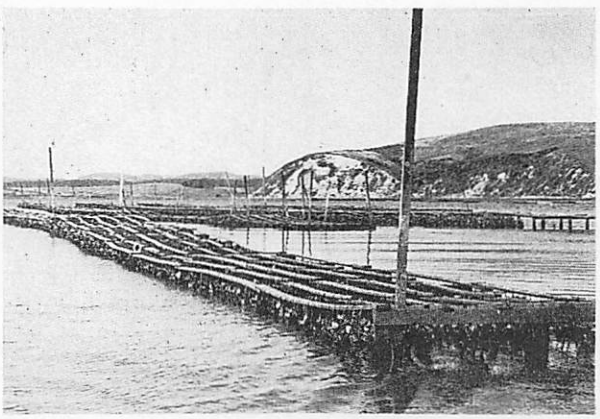


Figure 14. Commercial rack culture of Pacific oysters in Drakes Estero, California, March, 1970.

parasite (Figure 7) was found in low numbers but the incidence of infection exceeded 50% of the females observed in some months. Ancistrocomid ciliates (Figure 6) were observed in the digestive diverticulae of five to thirty percent of the oysters in the monthly samples. Trichodina like ciliates (Figure 8) was infrequently observed but were noted to infect 20% of the oysters in some monthly samples. The endoparasitic copepod *Mytilicola orientalis* was observed in 15 to 30% of the sample oysters. Larval cestodes *Echeneibothrium* sp., (Figure 9) were noted in the intestinal tract of approximately 2.5% of the oysters examined. This cestode was also observed in the intestinal tract and encysted in the tissues of clams in the area (Katkansky, Warner, and Poole, 1969).

European oysters (*O. edulis*) planted in Drakes Estero in 1963, 1964, and 1965 showed growth cessation and poor survival similar to plantings in Morro Bay and Elkhorn Slough. Histologically these oystere exhibited the same syndrome as those at Morro Bay and Elkhorn Slough and microcells were noted in 36% of the live oysters examined. As at the other two areas, nearby Pacific oysters were not infected.

6.4. Tomales Bay

One experimental station was established in Tomales Bay (Figure 13) in December 1966 and maintained through 1971. Losses sustained by the experimental populations (Table 13) varied from 7.8% to 35.9%. Histological examination of samples of oysters collected during the summer of 1967 when the highest mortalities occurred showed 30% of the gapers with bacterial foci (focal necrosis) shown in Figure 15. Similar conditions had been observed in Pacific oysters from Washington State and from Japan (Sindermann and Rosenfield, 1967) but the relation of these bacteria to the observed mortalities had not been established. Bacterial foci were observed from subsequent samples taken from Tomales Bay. However, prevalence never exceeded 5% of the gapers examined and few live oysters were observed with this condition.

Table 13. Percent mortality of 1964 to 1970 year class Pacific oysters at one station in Tomales Bay, California 1967 to 1971

Study year	Year class of oysters					
	1964	1966	1967	1968	1969	1970
1967	19.5	35.9				
1968	7.8	7.9	11.5			
1969			8.8	24.0		
1970			7.6	13.0[1]	13.9	
1971					22.8	11.1

[1] Experimental population inadequate; sample from commercial oyster bed.

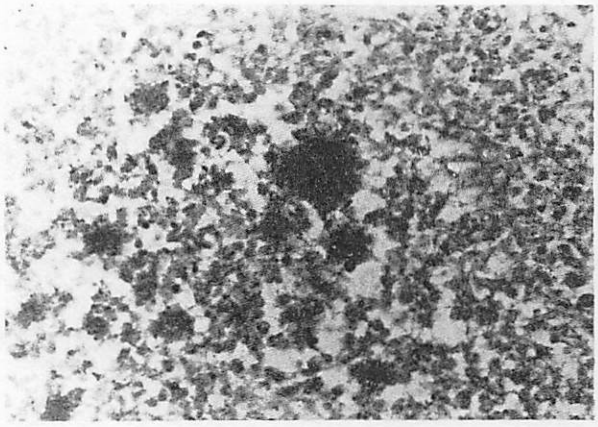


Figure 15. Bacterial Foci (Focal Necrosis) from gaping Pacific oyster from Tomales Bay, California, July, 1967.

Histological examination of oysters from Tomales Bay showed the same complement of parasites found in Morro Bay, Elkhorn Slough, and Drakes Estero although levels of infestation were not as high. Generally Ancistrocomid ciliates and *Mytilicola* infestation rates averaged less than 10%.

Adult American oysters (*Crassostrea virginica*) are periodically trucked from the Atlantic coast and held in Tomales Bay prior to sale in California markets. During routine histopathological monitoring, six American oysters (4 moribund and 2 living) infected by the haplosporidan, *Minchinia costalis* were observed in plantings of oysters which had been shipped from the vicinity of New Haven, Connecticut October 1966 and June 1967. Histological examination on adjacent stocks of Pacific oysters indicated no cases of *Minchinia* infection.

European oysters *O. edulis* were grown here as in Morro Bay, Elkhorn Slough, and Drakes Estero but survival in Tomales Bay was much higher than in the other three areas. After two and a half to three years, survival of stocks planted in 1963, 1964, and 1965 was 57%, 49% and 73% respectively. Grossly these oysters had the same general emaciated appearance observed in the other areas but no microcells were observed in the sample oysters.

6.5. Humboldt Bay

Natural reproduction of Pacific oysters does not occur in California so the industry depends upon the planting of seed oysters less than a year old originating from areas beyond the boundaries of the State.

For many years Japan supplied all the seed oysters used in California; however, in recent years significant amounts of seed collected in Washington State and British Columbia have been used. The seed was generally brought into the state in March or April and the mortalities which prompted this study began approximately 15 months later during the second summer after planting. During years of heavy losses significant mortalities also occurred among oysters in their third or fourth summers.

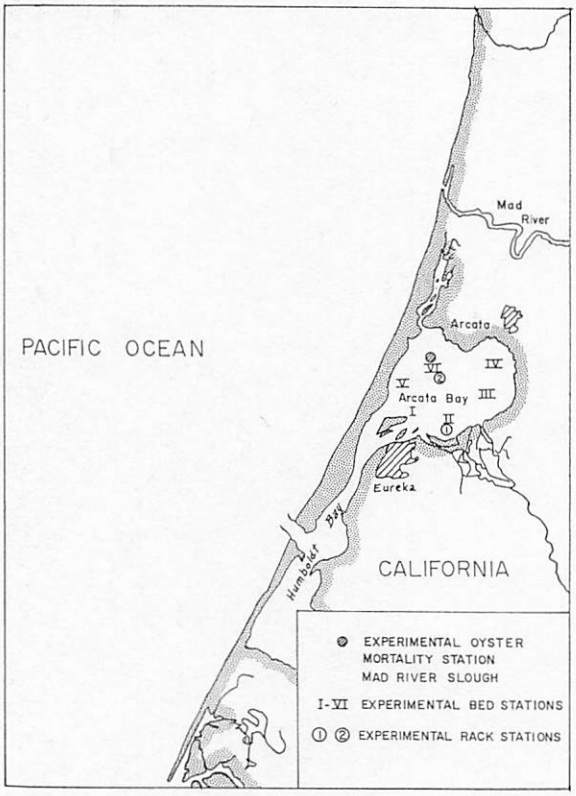


Figure 16. Location of experimental stations in Arcata Bay portion of Humboldt Bay, California

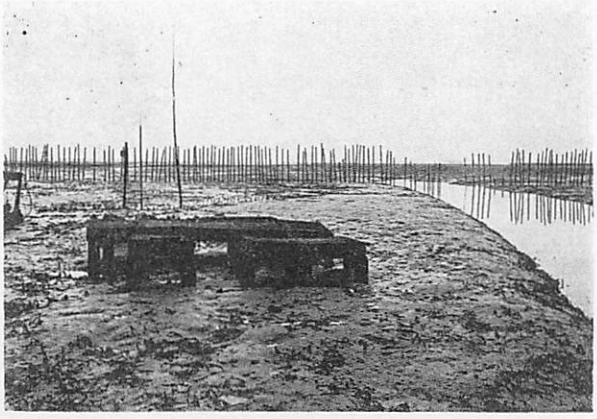


Figure 17. View of original experimental station in Humboldt Bay, December, 1971.

6.5.1. Mortality Rates

Seasonal losses among commmercial plantings of oysters during their second summer in Humboldt Bay ranged from 34.2% to 44.7% from 1961 through 1964 excluding losses from predation or other obvious causes. The maximum loss on a commercial bed during this period was reported as 56.4%. During the first summer of this study, 1966, losses among the 1965 commercial plantings averaged 33.4% and ranged from 12.4% to 55.9%.

One experimental station was established in Humboldt Bay on Mad River Channel in June, 1966 (Figure 16) and stocked with experimental populations of oysters placed in wire baskets as in the other California bays (Figure 17). This location was chosen because it was in an area of commercial culture containing oysters which were in their first and second summer so that comparisons between mortality of commercial and experimental populations were facilitated.

Mortalities observed among experimental populations in Humboldt Bay from 1966 to 1971 are shown in Table 14.

Losses for the 1965 seed planting during 1966 are shown as 21.8%; however, observations did not commence until July at which time the mortality period was well underway. Seasonal mortality was estimated at 40% compared to average loss among 1965 commercial plantings during 1966 which averaged 33.4%.

During the summers of 1967, 1968, and 1969, death rates among both experimental and commercial stocks showed marked declines from the 1961 to 1966 levels. Losses of 1966 year class commercial seed during 1967 were estimated at 10% to 15% while the comparable loss at experimental stations was 11.5%.

Table 14. Percent mortality of 1965 to 1970 year class Pacific oysters at a station on Mad River Channel, Humboldt Bay, California 1966 to 1971

Study year	Year class ofoysters					
	1965	1966[3]	1967	1968[2]	1969	1970[2]
1966	21.8[1]					
1967	21.5	11.5				
1968	8.3	9.7	12.6			
1969			9.6	6.2–14.2		
1970			25.9	10.1–25.8	20.	
1971				3.8–9.0	18.8	7.5–54.7

[1] From July on; Seasonal mortality about 40%.
[2] Range of mortality among lots from 5 seed sources.
[3] Seed from British Columbia.

Losses of 1967 year class commercial seed during 1968 ranged from 3.7% to 27.3% and averaged 16.1% compared to losses at the experimental station of 12.6%. Losses of 1968 year class commercial plantings durings 1969 averaged 12.0% compared to death rates among 5 experimental populations which ranged from 6.2% to 14.2%.

Losses during the summer of 1970 increased markedly over the previous three summers averaging 31.6% for commercial plantings during their second summer and 20% for comparable experimental populations. In 1971 losses among experimental plantings at six stations averaged 28.9% and ranged from 7.5% to 54.7%. Commercial plantings of seed in the spring of 1970 were inadequate to provide comparable year classes on commercial beds for comparison during 1970.

Commercial oyster culture using racks in the Mad River Slough area provided an opportunity to compare losses during 1970 on commercial racks, experimental trays, and commercial plantings on bottom. Mortality rates for rack-cultured oysters was 17.3% on the East side of Mad River Slough and 10.8% on the West side; losses in experimental trays were 20.0% and losses on bottom averaged 31.6%. In 1971 death rates among oysters cultured on the East Bay racks was 19.5%, at Mad River Slough 8.9%, and at Pantherati Slough 5.1% compared to losses in experimental trays at six locations which range from 7.5 to 54.7% and averaged 28.9%. A significant proportion of the deaths on racks was the result of predation by the rock crab (*Cancer productus*) but crab predation was not a factor among experimental oysters maintained in closed baskets. Threfore, the difference in mortality rates between rack culture and experimental plantings was greater than indicated.

The low mortality rate of rack-cultured oysters as well as rapid growth and excellent fatness suggested culture by this method, especially during periods of high mortality, even though the cause of the difference in mortality between rack and bed culture had not been determined.

6.5.2 Seed Source Study

Since Pacific oyster seed used by commercial growers originates in several areas, an experiment was designed and conducted to determine the effect of seed source on survival in high mortality areas in Washington and Humboldt Bay, California. Seed caught during the summer of 1697 at Mangoku-ura and Hojima, Japan, Willapa Bay, and Dabob Bay, Washington, and Pendrell Sound, British Columbia, was planted at Case Inlet, Washington and Humboldt Bay, California in May 1968. The Washington portion of this experiment is described in more detail in Section 4 of this report.

Five standard cases of seed from each of the five sources were planted in the Mad River Slough area of Humboldt Bay in May 1968 in rectangular plots 25′ × 135′. A space of 35′ was left between each plot

Figure 18. Seed source experiment, Humboldt Bay, California. Test strip showing ground planting and baskets.

Table 15. Pacific oyster seed source study. Mortality at Humboldt Bay, California 1968–1971

Seed source	Spat per shell May 15, 1968[1]	Spat per shell June 30, 1968	Mortality May 15–June 30, 1968 in percent	Spat per shell November 17, 1968	Mortality June 30–November 17, 1968 in percent	Spat per shell autumn 1969	Annual mortality 1969 in percent	Spat per shell autumn 1970	Annual mortality 1970 in percent	Spat per shell autumn 1971	Annual mortality 1971 in percent	Total annual mortality 1969, 1970, 1971 in percent	Total mortality after planting in percent
Willapa Bay	10.09	3.86	61.7	1.34	65.3	1.20	10.7	1.08	10.1	1.03	4.3	23.2	89.8
Mangoku-ura	7.10	3.95	44.4	2.19	44.6	1.99	9.0	1.48	25.8	1.40	5.4	36.1	80.3
Dabob Bay	56.69	32.31	43.0	18.48	42.8	17.00	8.0	14.57	14.3	13.38	8.1	27.6	76.4
Pendrell Sound	23.40	15.41	34.1	11.21	27.3	10.51	6.2	8.66	17.5	8.33	3.8	25.6	64.4
Hojima	7.94	6.29	20.8	4.83	23.7	4.11	14.2	3.26	20.6	2.97	9.0	38.0	62.6

[1] Differences between counts in Washington and California experiments is a result of losses during transit to California and during planting.

Table 16. Pacific oyster seed source study. Mortality at Case Inlet, Washington 1968–1971

Seed source	Spat per shell May 1968[1]	Spat per shell November 1968	Mortality May–November 1968 in percent	Spat per shell autumn 1969	Annual mortality 1969 in percent	Spat per shell autumn 1970	Annual mortality 1970 in percent	Spat per shell autumn 1971	Annual mortality 1971 in percent	Total annual mortality 1969, 1970, 1971 in percent	Total mortality after planting in percent
Willapa Bay	19	5	73.7	4.9	2.4	3.3	32.5	2.2	32.0	56.0	88.4
Mangoku-ura	13	7	46.1	6.0	13.8	3.9	33.6	2.3	40.0	67.1	82.3
Dabob Bay	80	20	75.0	19.3	11.3	11.3	41.4	4.9	57.0	75.6	93.8
Pendrell Sound	39	18	53.8	16.2	18.5	8.1	50.3	3.2	60.0	82.2	91.8
Hojima	14	6	57.1	4.1	31.9	3.2	21.1	2.4	23.5	60.0	82.9

[1] Differences between counts in Washington and California experiments is a result of losses during transit to California and during planting.

to allow definite separation of the stocks. A portion of the seed was placed in three experimental trays (Figure 18) adjacent to each plot to facilitate measurement of mortality. One hundred mother shells from each area were examined immediately after planting to determine the average spat count. Similar counts were made six weeks and approximately six months after planting to determine the pattern of loss. Observations during the late fall of 1969, 1970, and 1971 were made to determine seasonal mortality of oysters in each of the five groups (Table 15).

The initial average spat count per mother shell varied greatly among the five seed sources but could be separated into three groups: high count-Dabob at 56 spat per shell; medium count-Pendrell Sound at 23 per shell; and low count-Willapa Bay at 10 per shell, Hojima at 8 per shell and Mangoku-ura at 7 per shell. The relative order of these three groups continued throughout the experiment and at the end, Dabob Bay seed remained the highest with 13 spat per shell, Pendrell Sound next with 8 spat per shell and the other three sources at 1 to 3 spat per shell. The results of the Humboldt Bay experiment were similar to those of the Case Inlet, Washington experiment (Table 16). In Washington, average counts per spat per shell at the end of the experiment (November 1971) were 4.9 for Dabob Bay seed, 3.2 for Pendrell Sound seed, and 2.2 to 2.4 for seed from Willapa Bay, Mangoku-ura and Hojima.

The results of this experiment were difficult to interpret because of the great variation in initial spat count. However, seed from Hojima known as a high mortality area in Japan, had the highest losses during the second summer after planting in both California and Washington. In Humboldt Bay mortality of Hojima seed in the summer of 1969 was 14.2% compared to 6.2% to 10.7% for seed from the other sources. In Case Inlet, Washigton the mortality of Hojima seed during 1969 was 31.9% compared to 2.4%

Table 17. Growth of oysters from five seed sources planted in Case Inlet, Washington and Humboldt Bay, California May 1968 to December 1970

(Average volume in milliliters)

Experimental area	Seed source				
	Willapa Bay, Wash.	Pendrell Sound, B. C.	Hojima Japan	Dabob Bay, Wash.	Mangoku-ura Japan
Case Inlet, Wash.	144.3	115.3	143.3	131.7	139.0
Humboldt Bay, California	184.2	143.4	143.8	151.3	179.6

to 18.5% for seed from the other four sources. Seed from Mangouku-ura, known as a low mortality area in Japan, had losses during 1969 which were about average for the four sources other than Hojima. By the end of the experiments, however, seed from Hojima had about the same number of spat per shell as seed from Mangoku-ura and Willapa Bay.

The growth of oysters from the five seed sources after planting in Humboldt Bay, California, and Case Inlet, Washington, was determined by periodic measurements of average volume in milliliters, (Table 17). On the average, oysters grew more rapidly in California but with considerable variation among seed from various sources. In California, the seed from Willapa Bay and Mangoku-ura produced the largest oysters, 184.2 and 179.6ml. which were considerably larger than oysters from the other three seed sources which ranged from 143.4 to 151.3. In Washington, seed from Willapa Bay, Hojima, and Mangoku-ura produced the largest oysters, 144.3, 143.3 and 139.0ml respectively, whereas seed from the other two areas, Pendrell Sound and Dabob was considerably smaller, 115.3 and 131.7 respectively. It seems likely that Willapa Bay and Mangoku-ura seed produced larger oysters because this seed had fewer spat per shell which reduced competition for space and food. In

contrast, seed from Dabob Bay, Washington, and Pendrell Sound, British Columbia, with a larger number of spat per shell grew less rapidly.

It is interesting to note that oysters from each seed source except Hojima grew more rapidly in California than in Washington. Hojima seed had reached the same size in both locations by the end of 1979; however, this may have resulted from differential mortality rates together with the characteristic pattern of higher mortality among larger oysters.

6.5.3 Phytoplankton Study

In Washington, oystermen had observed that blooms of the dinoflagellate *Ceratium* coincided with the period of high oyster mortality in Southern Puget Sound. Investigations by Washington Department of Fisheries to determine the relation between *Ceratium* and oyster mortality are described in Section 4 of this report. Similar studies were conducted in Humboldt Bay, California, during the summers of 1967 and 1968. Washington Department of Fisheries participated in this study by analyzing water samples from Humboldt Bay to determine phosphates, nitrates, and chlorophyll A. The water of Humboldt Bay was found to be fairly rich in nutrients with a low standing crop of phytoplankton except during the spring. The high flushing rates in Humboldt Bay probably keep primary production at a fairly low level.

A preliminary phytoplankton survey during the summer of 1967 showed low abundance of dinoflagellates of the genera *Ceratium*, *Gymnodinium*, and *Peridinium*. The majority of the phytoplankton mass was composed of diatoms.

During the summer of 1968 an extensive phytoplankton study was carried out in Humboldt Bay. The number of dinoflagellates observed was extremely low and no blooms occurred. Oyster mortalities during 1968 were much lower than in previous years but it was not possible to determine the relationship between *Ceratium* and oyster mortalities from this experiment. The observation that *Ceratium* was scarce during the summer of 1967 when substantial oyster mortalities occurred in Humboldt Bay was similar to observations the same year in Case Inlet, Washington where high mortalities of oysters occurred while *Ceratium* was absent. Phytoplankton studies were discontinued after 1968 principally because of results of studies in Washington.

6.5.4 Histopathology-Humboldt bay

Samples of Pacific oysters were collected from experimental stations and from commercial beds in Humboldt Bay from 1968 to 1971 for histological examination to determine the presence of pathogenic organisms. For each year class a minimum of 10 live oysters and all of the dead oysters found at the time of sampling were removed each month from experiental stations. During the summer period when the high mortalities were expected, the sampling intensity was doubled. In addition samples of gaping (moribund) oysters were taken from commercial beds each summer beginning with 1968. The number of moribund oysters collected from commercial beds and examined histologically were as follows:

1968–380
1969–125
1970–614
1971–212

Microscopic examination revealed the same groups of parasites which were observed in oysters from other California bays. *Mytilicola orientalis* was the most commonly observed parasite, occurring in up to 25% of the oysters examined each month (Katkansky, Sparks & Chew, 1967). The usual site of infestation was the intestinal tract but during 1967 unusual incidences of infestation in the digestive diverticulae were observed. A marked host response indicated by leucocytic infiltration occurs when this parasite is found in the digestive diverticulae. This response can cause resorption of the parasite (Katkansky and Warner, 1968).

Other parasites included Ancistrocomid ciliates which were observed in the digestive diverticulae in less than 10% of the oysters observed and less frequently in the gills and palps. Ciliates of the genus *Trichodina* were found occasionally on the gills and palps of oysters. Unidentified ovarian parasites were noted in up to 60% of the female oysters collected during the early summer months. Larval cestodes genus *Echeneibothrium* were found in the intestine of less than 0.1% of the oysters examined but no encysted pleocercoid larvae were observed.

Histological examination of oysters from Humboldt Bay disclosed a condition described as "inclusion cells" which had not been observed in oysters from other California bays (Figure 19). Inclusion cells were noted in an average of 6% of the moribund oysters and usually in 10% or less of the live oysters although on one occasion inclusion cells were observed in 21% of a sample of live oysters.

The precise nature of the condition described as "inclusion cells" has not been resolved. Some of the pathologists involved in the West coast oyster mortaliy investigations considered this so-called Humboldt Bay organism to be the etiological agent responsible for mass mortalities of oysters in the area. Others considered that the condition described as

"inclusion cells" was not in fact an organism but phagocytic oyster cells. Further study beyond the scope of the present investigations will be needed to solve this controversy.

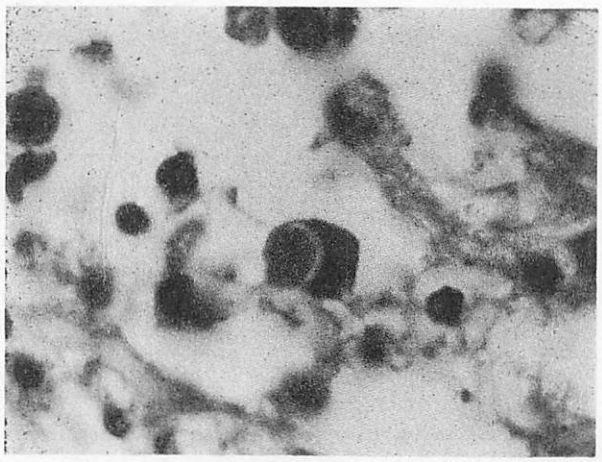


Figure 19. Inclusion cells in *C. gigas* from Humboldt Bay.

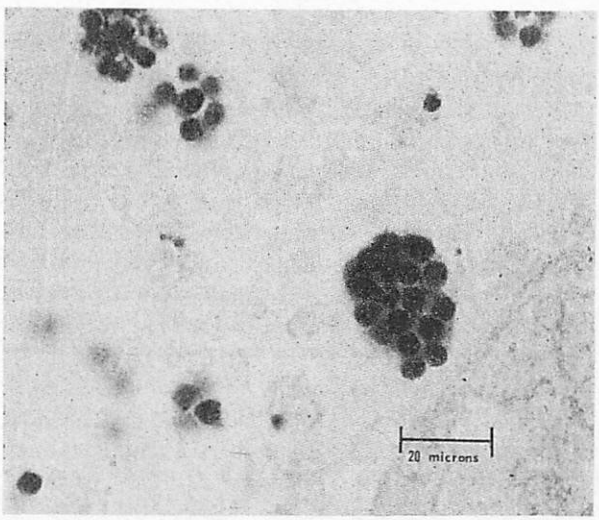


Figure 20. Haplosporidan spores in *C. gigas* from Humboldt Bay, California, 1969.

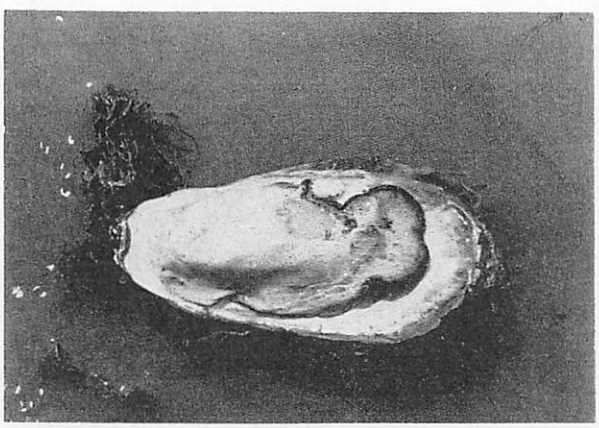


Figure 21. Dead oyster *Crassostrea gigas* from commercial beds in Humboldt Bay, June, 1970.

Characteristically moribund oysters in Humboldt Bay are large for their age and the meats are well developed with high levels of gylcogen or gonadal and can be described as being "in good coudition" (Figure 21). In contrast, one Pacific oyster from a seed planting in 1966 was examined in August 1969 and found to be thin and watery and in an emaciated condition. Histological examination of tissues from this oyster after staining with a technique described by Farley, 1965, showed this oyster to be infected by acid-fast operculated spores which were provisionally identified as belonging to the genus *Minchinia* (Figure 20). Sporulation resembled that of *Minchinia costalis* for it occurred in the Leydig tissue but the spores appeared intermediate in size between *M. costalis* and *M. nelsoni* (Katkansky, Warner, 1970). This apparently was the first recorded instance of sporulation of a haplosporidan in *Crassostrea gigas* on the West coast of the United States. Since this condition was observed in only individual and since the gross appearance of this oyster differed greatly from the characteristic appearance of moribund oysters in Humboldt Bay it is highly unlikely that this haplosporidan was the cause of mass mortality of oysters in this area.

Pathological studies of Humboldt Bay oysters did not include an investigation of the role of bacteria in oyster mortalities. Near the end of the study period laboratory experiments at the University of Washington indicated the relation between bacteria believed to be of the genue *Vibrio* and mortality of Pacific oysters at elevated temperatures (Lipovsky and Chew, 1972). Lack of funding terminated the West coast oyster mortality investigations before studies could be conducted in Humboldt Bay to determine the relation between *Vibrio* and oyster mortalities.

7. HISTOPATHOLOGICAL STUDIES-GENERAL

Histopathological studies were conducted by four groups, Battelle Northwest, University of Washington, Oxford Laboratory of the National Marine Fisheries Service, and California Department of Fish and Game. Findings of Dr. Stanley Katkansky, pathologist for California Department of Fish and Game and his associates have been described in Section 6 of this report.

7.1 Battelle Northwest

During the period from May 1966 to May 1968 a research team of the Northwest laboratory of Battelle Memorial Institute at Richland, Washington con-

ducted research to evaluate various histopathological techniques which might be of value for examining oysters in search of the cause of mass mortalities. During this period the team, headed by Dr. Gilbert Pauley, investigated the applicability of electron microscopy, described fixation techniques applicable to oyster tissues, and investigated the histochemistry of oysters especially related to changes in glycogen and lipid distribution when infected by micro-organisms. The team also described 22 special staining techniques and discussed the applicability of each to oyster pathology. Four of these techniques were found to have applicability to oyster tissues and certain related mollusks (Pauley, 1967; Pauley and Maulsby, 1967; Heaton and Pauley, 1969). Additionally, a method of fixation that had use in histochemical staining was described (Pauley and Clarke, 1967).

During the course of the research, the Battelle team prepared and examined several hundred slides from oysters in Willapa and Humboldt Bays. Several types of tumors observed during these studies, and reported in the literature, include a watery cyst (Pauley and Sayce, 1967, and Pauley et al., 1968), an internal fibrous tumor (Pauley and Sayce, 1968), a mesenchymal tumor (Sparks, et al., 1968), and a nerve tumor (Pauley, et al., 1968).

Pauley and Becker also studied the unidentified parasite in the maturing ova of Pacific oysters *Crassostrea gigas* from Humboldt Bay. This organism differed in many respects from known parasites of bivalve mollusks and consequently its systematic position remained problematical. Superfically, because of its characteristic infection site, it resembled *Chytridiopsis ovicola* and *C. mytilovum* found in the European oyster, *Ostrea edulis* and the bay mussel, *Mytilus edulis* (Becker and Pauley, 1968).

7.2 University of Washington Studies

Pathologists of the University of Washington College of Fisheries, under the direction of Dr. Albert K. Sparks and Dr. Kenneth K. Chew, participated in the West coast oyster mortality investigation from 1966 to June 30, 1970. The function of the College of Fisheries was the preparation of slides from sample oysters provided by State fisheries agencies and examination of tissues for micro-organisms suspected as pathogens. An analysis of the results of these studies and representative slides were returned to the State agencies and sent to the National Marine Fisheries Service Laboratory at Oxford, Maryland in exchange for similar slides prepared by that laboratory.

The usual parasites of oysters of the genera *Ancistrocoma,* and *Mytilicola* and Trichodina-like ciliates were found at various levels of incidence in most oyster samples. Although these parasites are usually rejected as possible causes of mortality, there are some resasons to consider members of the genus *Mytilicola* as a possible disease factor (Chew, et al., 1971). Investigations into early life history of *Mytilicola* sp. indicate that the parasitic larval reside in the stomach and large collecting ducts of the digestive gland where they maintain their position by attaching to the epithelium with their hooked antennae. In this location parasites are most likely to compete with the host for ingested food passing to the digestive gland. The means of attachment also probably causes small lesions which would permit secondary bacterial infections. The possibility of adverse effects from the larvae of *Mytilicola* had not been considered in previous studies and do not show up in routine histological examination. Although larval *Mytilicola* appear to be capable of producing harmful affects, there is at present, no evidence to indicate that they are responsible for the heavy mortalities of oysters in some parts of Washington.

Foci of bacteria were found infrequently in Oregon oysters which generally show few pathological symptoms as might be expected because of the extremely low mortality rates of Pacific oysters in Oregon. In contrast foci of bacteria were found frequently in oyster samples from Eld Inlet, a heavy mortality area in Southern Puget Sound, Washington. The University of Washington participated in the floating live box studies in high, medium, and low mortality portions of Eld Inlet by examining samples provided by the Washington Department of Fisheries. Histological examination of these oysters indicated incidence rates for bacterial foci of five to ten pecent; however, incidence did not correlate with mortality rates observed at the three experimetal locations.

Oysters from the Eld Inlet livebox experiment showed other abnormal conditions. Tissue necrosis was observed in 17% of the oysters examined from the high mortality location E–1, 13% from the intermediate mortality location E–2, and 4% from the low mortality area E–3 during 1968. These oysters were characterized by leucocytic infiltration of Leydig tissue, in some cases localized in portions of the gonad. This was often accompanied by lysis of the Leydig tissue in various parts of the oyster and increases in the size of pigment granules. Necrosis and hemocytic infiltration in the gonad was usually found in female oysters, but also occurred in some males. Usually this condition was accompanied by numerous foci of bacteria in the gonads and at times in other parts of the oyster. Although at first foci of bacteria appeared to be the primary pathogen, examples of this pathological condition were found in which no foci of

bacteria were observed.

Oyster samples from the three floating live boxes in Eld Inlet in 1969 had moderate to heavy leucocytic (hemocytic) infiltration of the gill tissues. This did not appear to be due to resorption of gonadal products as this process had not yet begun in many oysters. In cases where foci of bacteria were present, this leucocytic Infiltration extended throughout most of the tissues. The agent stimulating the leucocytic infiltration of the gills could not be determined from histological preparations. The response of the oyster may have been due to some factor too small to be seen with a light microscope such as a small bacterium or virus. Another possiblity is that the response of the oyster was because of a pollutant or other environmental factor which would cause gill tissue to be affected first. Leucocytic infiltration of the gills was observed in 66% of the sample oysters from the high mortality portion of Eld Inlet float E–1, 44% at the intermediate mortality station float E–2, and 35% at the low mortality station E–3 during 1969.

In summary two conditions regarded as pathological: tissue necrosis with or without detectable bacterial foci, and leucocytic infiltration of the gills were observed in experimental oysters from Eld Inlet, Washington, and appeared to correlate with the observed mortality rates at three floating live box stations.

In a project which predated the West coast oyster mortality investigations, Dr. Albert K. Sparks and his associates studied oysters from Humboldt Bay, California in an attempt to determine the cause of extensive mortalities which had been reported by commercial oystermen during the period 1962 to 1964. Mortalities as high 50% had been reported during a mortality period beginning in July and continuing into September. Oyster samples from experimental plantings at intertidal locations and in floating livecars maintained by the University of Washington team from 1963 through 1967 were examined for pathogenic organisms. An amoeba, *Vahlkampfia* sp. was found in oyster samples from Humboldt Bay and was believed to be responsible for the oyster mortalities. This organism was first observed in oysters by Hogue (1914, 1921). Sparks, et al. (1967, 1968) reported identification of several stages in the life cycle of *Vahlkampfia* without elucidation of the complete life cycle. One stage of the life cycle, an inclusion cell, was reported to be particularly conspicuous, abundant, and diagnostic of the disease in Humboldt Bay. Subsequently, Katkansky found inclusion cells (Figure 19) in high percentages of moribund oysters in Humboldt Bay, as discussed in Section 6 of this report. The role of inclusion cells in the life cycle of *Vahlkampfia* and their relation to oyster mortality in Humboldt Bay has not been determined.

7.3 Studies at National Marine Fisheries Service Laboratory, Oxford, Maryland

As the primary pathology laboratory for the National Marine Fisheries Service, the Oxford, Maryland laboratory participated in the West coast mortality investigations by examining fresh and preserved samples of oysters and associated species from Washington, Oregon, and California to determine the presence of micro-organisms suspected as pathogens. As a general rule, samples taken in Oregon were shipped directly to the Oxford laboratory for processing. Samples from Washington generally were sent to the University of Washington for preparation of slides and representative samples were forwarded to the Oxford, Maryland laboratory for comparative studies. In general, California samples were processed by the pathologist of the California Department of fish and Game, but representative slides were frequently referred to the Oxford, Maryland laboratory for verification or for further study. In addition, the Oxford, Maryland laboratory examined oysters from Canada and from several seed areas in the Orient.

Research priority at the Oxford Laboratory was given to potential pathogens which were suspected to be related to mass mortality of oysters in Washington and California. Two principal conditions studied were termed focal necrosis and microcell disease.

7.3.1 Focal Necrosis

A pathological condition, presumably the same as that called "multiple abscess" by Japanese workers, was observed in oysters from high mortality areas in Matsushima Bay, Japan and Willapa Bay and Southern Puget Sound, Washington. The disease, apparently caused by a bacterium, appears as necrotic foci deep within the tissues. Rapid diagnostic methods were developed using gross pathology signs and ultra-violet microscopy. This permitted screening of a relatively large number of live oysters from the Naselle River area in Willapa Bay, Washington and from Matsushima Bay in Japan.

The etiologic agent of focal necrosis appears to be a gram-positive diphtheroid-like microorganism occurring in foci, often widespread, throughout the tissues. Squashes sometimes reveal the rodlike organisms spilling out of the foci. Many attempts were made to isolate the organism contained within the foci using a number of special media under a variety of environmental conditions. Microbiologists at the Oxford Laboratory were not successful in isolating the organism although isolations of other bacterial

forms were made from these materials.

Histopathological studies of Willapa Bay oysters indicated a high prevalence of focal necrosis (30%) during a heavy mortality period in April 1965. Samples taken in December, 1966 January, 1967, and March, 1968, from Willapa Bay during periods of low mortality had low incidence of focal necrosis (5%, 4%, and 8% respectively).

The occurrence of mantle recession was found to be a reliable gross indication of disease in Pacific oysters from Willapa Bay. In fresh tissue squashes, bacteria in foci fluoresce green against the red tissue during ultra-violet microscopy (G. E. Krantz, personal communication). Fresh tissues of infected oysters also show abnormal concentrations of hyaline hemocytes and infection can be quickly verified using a modified gram stain that employs wet fixation and alcohol dehydration of squashes. Stained tissue sections have indicated that focal necrosis organisms, in addition to being gram-positive, are PAS positive, and moderately acid-fast (Farley, 1965).

7.3.2 Microcell Disease (Denman Island Disease)

Although exact characterization of this disease is still in question, it appears that "microcells" occur in *Crassostrea gigas* from Denman Island, British Columbia, hence the name. Infection by microcells appears to occur in the epithelia of the gill and alimentary canal. In *C. gigas* the disease is characterized by mantle recession and by local abscesses in the vesicular tissue.

In *Ostrea edulis* local lesions in the epithelia are followed by systemic invasion of all tissues accompanied by infiltration, lysis and necrosis. Microcells were found in tissues of *O. edulis* from Milford, Connecticut, which had been kept in Chincoteague Bay, Virginia for several months. They were also found in tissues of *O. edulis* from Milford which had been introduced to Morro Bay, Elkhorn Slough, Drakes Estero, and Tomales Bay, California. Also, microcell disease developed in *O. ednlis* from Milford, which were being held in recirculated sea water aquaria at the Oxford, Maryland laboratory.

In an attempt to understand the role of microcells in the mortality of *C. gigas*, a collaborative study was initiated between Oxford, Maryland Laboratory and the Fisheries Research Board of Canada. This study was based on the shipment of 25 Denman Island oysters to Oxford, Maryland each month for a period of 12 months to facilitate in *vitro* culture studies. During these studies, an amoeboid organism was isolated from Canadian oysters.

In addition, samples of the native Australian oyster, *Crassostrea commercialis* (*gromerata*) from Wooloowarre Bay, Australia, a high mortality area, were found to be heavily infected with a "microcell" organism. The organism involved appears to be identical in *O. edulis* and *C. verginica*, but differs in *C. commercialis* from Australia in that the nucleus is peripheral to slightly eccentric compared to the centrally located nucleus in microcells in the other two species. The discovery of microcell organisms in Australian oysters suggests the possibility that this may be the causative agent of the Australian winter disease reported by Roughley (1962) and more recently, by Peter Wolf (personal communication to C. A. Farley). Examination of samples of *O. edulis* from Milford, Connecticut, which had experienced high mortality at Pigeon Point, California, Morro Bay, California, and Chincoteague Bay, Maryland indicated high prevalence of microcell disease (83%, 80%, and 50% respectively). Examination of another group of these oysters which had experienced a 40% mortality at Morro Bay indicated a 30% prevalence of microcells. No microcells were found in samples of oysters from Tomales Bay, Morro Bay, and Chincoteague Bay, which had low or no mortality. Samples of *Crassostrea gigas* from Denman Island, British Columbia, which had experienced low mortality indicated an 18% prevalence of microcells.

Although it appears probable that "microcells" are involved in the mortality of three species of oysters the identity and life history of the causative organism have not been established.

It is interesting to note that *C. gigas* adjacent to *O. edulis* heavily infected with microcells in Morro Bay, Elkhorn Slough, and Drakes Estero, California were not observed to be infected by microcells.

7.3.3 Neoplasms

An unusual histopathological condition of the native oyster *Ostrea lurida* from Yaquina Bay, Oregon was reported by Jones and Sparks, 1969. In 1967 C. Austin Farley discovered this disorder in duplicate slides of Yaquina Bay *O. lurida* supplied by the University of Washington College of Fisheries. In September, 1968, a neoplastic condition resembling the *O. lurida* disorder was found in 7% of *Mytilus edulis* collected by staff of the Oregon Fish Commission. Subsequent samples in January, 1969 revealed a 12% prevalence of the disorder in bay mussels and native oysters. Papers by Farley, 1969, and Farley & Sparks, 1969, described this disease.

According to C. Austin Farley, the neoplastic disorder in *O. lurida* is easily recognized microscopically, even in unstained living tissue squash preparations. Neoplastic cells are about 12 microns in diameter. The cytoplasm is hyaline and the cells are amoeboid,

moving with stellate to lobate pseudopodia; however, motility is much slower than that of granular phagocytes. In histologic sections, these cells stand out due to the abnormally large (5 to 8 micron), round to lobate, dense, basophilic staining nucleus. A large necleolus is present in most neoplastic cells and some contain a yellowish inclusion which often appears to have spoke-like processes. Binucleated and multinucleated forms are seen occasionally. Mitosis can usually be seen in affected oysters; however, it is not found as abundantly as in mussel neoplasms.

Several stages of the disease are recognized histopathologically. The earliest stage is characterized by the appearance of a few neoplastic cells in isolated locations, generally in the vesicular connective tissue. Enlarged vesicular cell nuclei can usually be found and neoplastic cells sometimes appear to be collecting up vesicular connective tissue cells. The origin of these neoplastic cells seems to be multifocal. No gross pathology is evident in this stage.

Intermediates stages show moderately diffuse disseminations of neoplastic cell infiltrations in hemolymph spaces and the vesicular connective tissue. Gross pathology may be evident in the form of pale digestive gland or poor condition.

Advanced cases show massive invasion of the connective tisssue, gonad, and hemolymph with complete arrestment of gametogenesis. Poor condition, pale digestive gland, and mantle recession are usually present in this stage and are the gross indications of pathology. Terminal cases show degenerative changes in neoplastic cells such as breakdown of the nuclear membrane, karyolysis, pyknosis, and general necrosis of tissues. Gross tumors have been found in only two of the fifty cases studied and were probab ly the result of occlusion rather than true solid tumor formations, because both had massive disseminations and showed little reticulin or collagenous material in the stromas of the respective tumors.

The neoplastic disease has been diagnosed from oysters *O. lurida* collected in every month but March, June, and November, which probably indicates inadequate sampling rather than absence of the condition. The disease appears to be most prevalent in the fall and early winter with the most advanced cases occurring in early winter. The highest prevalence of neoplastic disease in *O. lurida* yet noted was recorded from a sample collected in late September, 1969, when 40% of the oysters were affected by this disorder.

Although this disorder does not involve *Crassostrea gigas* as far as is known, it is of considerable interest because of its prevalence in Yaquina Bay, Oregon and the general interest in leukemia-like infections.

8. DISCUSSION-CONCLUSIONS

The six year study provided a good description of the pattern of oyster mortalities on the Pacific coast of the United States. Locations of high mortality were identified, and the seasonal occurrence of high mortality of fast growing, fat oysters during their second summer near the head of productive estuaries, was described. Low mortality areas were characterized as having less turbidity, lower productivity, and slow-growing, thin oysters.

Several parasites and other potentially pathogenic

Table. 18. Incidence of parasites and other potentially pathogenic conditions observed in Pacific oyster samples from Washington and California, 1965-1972

	Incidence: percent of oysters examined								
Location	Ancistrocoma	Trichodina Like Citiates	Mytilicola	Ovarian parasite	Bacterial Foci (Focal Necrosis)	Tissue Necrosis	Leucocytic Infiltration of Gills	Inclusion Cells	Minchinia sp.
Eld, Inlet, Washington	11-25	Occasional	4-17		4-5	4-17	35-66		
Willapa Bay, Washington					4-30				
Morro Bay, California	Up to 12%	Occasional	25-40	Low[1]					
Elkhorn Slough, California	Up to 10%	Occasional	5-15	12-40					
Drakes Estero, California	5-30	20	15-30						
Tomales Bay, California	Up to 10%		Up to 10%		Up to 30%				
Humboldt Bay, California	Up to 10%	Occasional	Up to 25%	Up to 60%				6-20	One oyster

[1] Except 60% in one sample.

conditions were observed in Pacific oysters during the course of the studies, as summarized in Table 18. Ciliates of the genus *Ancistrocoma* (Figure 6) were observed in the digestive diverticulae of up to 30% of the oysters examined. There appeared to be no correlation between the incidence of this ciliate and mass mortality. Peritrichous Trichodina-like ciliates (Figure 8) were noted on the gills and mantles of an occasional Pacific oyster from most areas, but were not considered as pathogens.

The endoparasitic copepod, *Mytilicola orientalis* was found in oyster samples from most areas at incidence levels up to 40%. Although these parasites are usually rejected as a possible cause of mass mortalities there are some reasons to consider *Mytilicola* sp. as a possible disease factor. Larval stages reside in the stomach and large collecting ducts of the digestive gland where they maintain their position by attaching to the epithelium with their hooked antennae. In this location they are likely to compete with the host for ingested food passing to the digestive gland. Additionally, the means of attachment causes small lesions which could permit secondary bacterial infections. Although the larval *Mytilicola* are capable of producing harmful effects, there is at present no evidence to indicate that they are responsible for the heavy mortalities of oysters observed during these studies.

An unidentified ovarian parasite (Figure 7) was observed in female oysters but was not considered to be a cause of mortality nor to have a serious effect on reproduction because of the low incidence of infection. This organism differs in many respects from known parasites of bivalve mollusks and consequently its systematic position remains problematical. Superficially, because of its characteristic infection site, it resembles *Chytridiopsis* sp. found in the European oyster, *Ostrea edulis*, and the bay mussel, *Mytilus edulis* (Becker and Pauley, 1968).

Bacterial foci of focal necrosis, a pathological condition, presumably the same as that called "multiple abscesses" by Japanese workers was observed in oysters from high mortality areas in Willapa Bay and Southern Puget Sound, Washington. The disease appears as necrotic foci deep within the tissues and the etiologic agent appears to be a gram-positive diphtheroid-like microorganism occurring in foci, often widespread throughout the tissue. Attempts to isolate the organism contained within the foci were unsuccessful. Bacterial foci were infrequent in Oregon, where mortality rates were low, but were common in oyster samples from Eld inlet, Washington, a high mortality area.

A condition described as "tissue necrosis" was observed in oysters from the floating livebox experiments in Eld Inlet, Washington. Usually this condition was accompanied by numerous foci of bacteria in gonads and at times in other parts of the oyster. Although at first foci of bacteria appeared to be the primary pathogen, examples of this pathological condition were found in which no foci of bacteria were observed. Oysters affected by tissue necrosis were characterized by leucocytic infiltration of Leydig tissue which was often accompanied by lysis of Leydig tissue in various parts of the oyster and increase in the size of pigment granules. Because of the occurrence of this condition in the absence of bacteria it was described separately from focal necrosis or bacterial foci.

Another condition observed in oyster samples from the three floating liveboxes in Eld Inlet in 1969, was a heavy leucocytic (hemocytic) infiltration of the gill tissues. The agent stimulating the leucocytic infiltration of the gills could not be determined from histological preparations. This condition was observed in 66% of the sample oysters from the high mortality station of Eld Inlet, 44% at the intermediate mortality station, and 35% at the low mortality station. Both this condition and the tissue necrosis, with or without detectable bacterial foci appeared to correlate with observed mortality rates at the three floating livebox stations.

Histological examination of oyoters from Humboldt Bay disclosed a condition described as "inclusion cells" which had not been observed in oysters from other California bays (Figure 19) but the precise nature of this condition was not resolved. In an earlier study, Sparks, et al., reported identification of several stages of the life cycle of the amoeba *Vahlkampfia* from Humboldt Bay without elucidation of the complete life cycle. One stage of the life cycle, an inclusion cell, was reported to be particularly conspicuous, abundant, and diagnostic of the disease in Humboldt Bay. The role of inclusion cells in the life cycle of *Vahlkampfia* and their relation to oyster mortality in Humboldt Bay have not been determined.

One Pacific oyster from Humboldt Bay was found to be infected by acid-fast operculated spores which were provisionally identified as belonging to the genus *Minchinia* (Figure 20). This apparently was the first recorded instance of sporulation of a Haplosporidan in *Crassostrea gigas* on the West coast of the United States. Since this condition was observed in only one individual, and since the gross appearance of this oyster differed greatly from the characteristic appearance of moribund oysters in Humboldt Bay, it was considered highly unlikely that this Haplosporidan was the cause of mass mortality of oysters in

this area.

In summary, only three of the conditions listed in Table 18 appeared to be important in relation to the observed mass mortality on the Pacific coast of the United States. These were bacterial foci (focal necrosis), tissue necrosis, and leucocytic infiltration of the gills. The possibility that these conditions had resulted from a bacterial infection cannot be rejected because only limited bacteriological studies were conducted during the course of the investigations. Likewise, no virological studies were conducted so the possibility of virus diseases cannot be ruled out.

Research, which began at the University of Washington about the time this investigation was completed, implicated bacteria such as *Vibrio* in the mortality of oysters at elevated temperatures. There is a possibility that some or all of the observed oyster mortalities were caused by *Vibrio* or similar bacteria.

The occurrence of the mortality during the midsummer spawning period led to the physiogical stress theory which has also been used to explain the cause of oyster mortalities in Matsushima Bay, Japan. The failure of these investigations to find a causative organism tends to strengthen the physiological stress theory, although additional research would be needed to describe the precise nature of this condition and the factors which result in the death of oysters.

The theory that oyster mortalities were related to blooms of the dinoflagellate *Ceratium* sp. was disproved when heavy mortalities occurred at Case Inlet, Washington in the absence of this organism.

Experimental planting in Washington and California of seed from five sources failed to disclose differences in survival attributable to diseases or disease resistance. Seed from Hojima, Japan, known as a high mortality area, had the highest losses during the second summer after planting in both California and Washington. Seed from Mangoku-ura, Japan, known as a low mortality area, had losses which were about average for the four sources other than Hojima. By the end of the experiment, however, seed from Hojima had about the same number of spat per shell as seed from Mangoku-ura and Willapa Bay.

It is interesting to note that oysters from each seed source except Hojima grew more rapidly in California than in Washington. Hojima seed had reached the same size in the two locations by the end of the experiment.

Except for Willapa Bay, there appears to be little likelihood of developing immune strains among local populations by natural selection. Mass mortalities have not been observed in Dabob Bay or Pendrell Sound, the two principal North American seed sources. Successful repoduction occurs sporadically in Willapa Bay and certain parts of this area have experienced heavy mortalities of oysters. If survivors remain in areas which become warm enough to stimulate spawning, a certain degree of disease resistance might be established through genetic selection.

If the mortalities reported from Matsushima Bay, Japan are caused by the same organism as those on the U.S. West coast there is a possibility that disease resistant seed will develop naturally.

The possibility remains of using survivors of oyster mortalities in Southern Puget Sound or Humboldt Bay as spawning stock for local oyster hatheries. This technique is currently being tried at the California Department of Fish and Game, Granite Creek Laboratory. Recent success of Virginia Institute of Marine Sciences in developing strains of *Crassostrea virginica* resistant to *Minchinia* infections would encourage similar attempts on the Pacific coast.

Efforts by the Washington Department of Fisheries to improve oyster culture techniques to more than offset the adverse affects of oyster mortalities appear to be productive. Already they have developed procedures for increasing survival of seed oysters during the first few months after planting and application of these techniques should be of great assistance to the oyster industry.

A decreasing trend in oyster mortalities has been observed during 1972 and 1973 after termination of the study, and it is possible that mass mortalities of Pacific oysters like many changes in animal populations will prove to be cyclic.

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PRESENT STATUS OF STUDIES ON THE MASS MORTALITY OF CULTURED OYSTERS IN JAPAN AND ITS PREVENTION

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INTRODUCTION

Today I would like to talk briefly about the present status of the studies and prevention of oyster mass mortality in Japan.

Several years ago, we including the late Dr. T. Imai, were offered a proposal of co-operation on studies of the oyster mass mortality by friends in the United States, but unfortunately it was unworkable for various reasons.

As you know, the techniques of oyster culture in Japan and the United States are quite different, but the same species of oyster is produced in both countries. So, the study of oyster mass mortality is common interest and continues to be a very important problem in both countries.

THE BACKGROUND AND PRESENT STATUS OF THE MASS MORTALITY PROBLEM

The Pacific oyster was cultured by the stick and sowing methods, for more than two centuries at Hiroshima without any notable modifications.

In 1923, the hanging method was applied practical oyster culture. After that, the hanging method was rapidly extended to various areas, and extensive oyster culture grounds were completed at Hiroshima, Miyagi, Shizuoka, Iwate, Wakayama, Shimane, Kumamoto, and Mie Prefectures.

At the present time, five different methods: raft, longline, rack, stick and sowing methods, are applied depending on the character of the culturing grounds. The three hanging methods, raft, longline and rack, are the most important and popular in Japan. Raft and rack methods are used principally in the southern part of Japan, but the longline method is commonly used along the coast of the Tohoku region.

Because of the adverse conditions caused by water pollution, the productivity of most oyster grounds located in shallow areas decreased. To avoid these

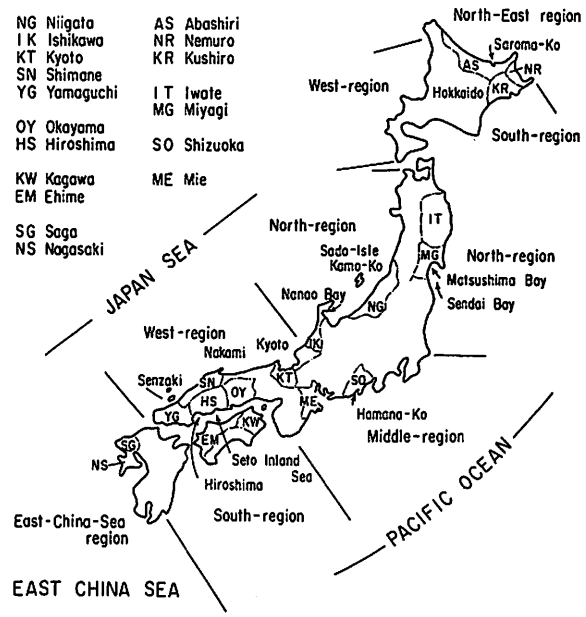

Figure 1. Map showing major oyster culture grounds in Japan.

situations, oyster grounds are being moved to offshore area which have relatively high productivity.

The hanging method is the most effective culturing method, but from the begining it has been accompanied by mass mortality. Recently mass mortalities spread over all culture grounds in Japan. At present, mass mortalities appear more often on Pacific coast, especially Hiroshima and Matsushima Bay, than on the Japan Sea side farms.

The causes of this misfortune have not been clarified, but it was generally said the major causes of mass death of oysters were impeded spawning due to high water temperature and high salinity during the spawning season and pollution of the beds.

THE PATTERN OF MASS-DEATH

In general, the causative agents of mass mortality of shellfish have been classified into following types,

1) Abnormally high water temperature and high

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salinity,

2) Abnormally low water temperature and low salinity,
3) Physiological impediment by high water temperature or high salinity,
4) Insufficient insolution and consequent shortage of food,
5) Parasites (Polydora, Hydroides, Flat worm),
6) Pollution,
7) Disease,
8) Pathogenic micro-organisms.

In Japan, most of the researchers have estimated that 3), 5), 6) and 8) are the most lethal causes of oyster mass death. But these causative agents are related closely not only with each other, but with physico-chemical environments.

There has been a striking resemblance between the mass mortality of the cultured oyster which occurred in Hiroshima and that in Matsushima since 1945. The following points were common to both areas.

1) Mass-death occurs during the spawning season in most of the cases, but in some regions after the spawning.
2) One-year or older oysters suffered mass death.
3) Motality rates are higher in fast-growing and larger oysters.
4) Significant differences in hydrographical conditions can not be found between years of mass-death and usual years.
5) Mass-death of oysters begins with the rise of

Table 1. Records of mass-mortality of oyster and its estimated cause in Japan

	Japan Sea coast					Pacific Ocean Coast		
	Kamo-ko Niigata-Pref.	Nanao-Bay Ishikawa-Pref.	Kyoto Kyoto-Fu.	Nakami Shimane-Pref.	Senzaki Yamaguchi-Pref.	Matsushima Miyagi-Pref.	Hamana-ko Shizuoka-Pref.	Hiroshima Hiroshima-Pref.
1945	—	—	—	—	—	—	—	—
1946	—	—	—	—	—	—	—	●
1947	—	—	—	—	—	—	—	●
1948	—	—	—	□	—	—	—	○
1949	—	—	—	○	—	—	—	—
1950	—	—	—	○	—	—	◎ B	—
1951	◎	—	—	—	—	—	◎ A	—
1952	◎	—	—	—	—	—	○	—
1953	—	—	—	—	—	—	—	○ C
1954	—	—	—	—	—	—	○	○ C
1955	—	—	—	—	—	—	—	—
1956	—	—	—	—	—	—	○	—
1957	—	—	—	—	—	—	◎	—
1958	—	—	—	○	—	◎	◎	—
1959	—	—	—	○	—	—	○	—
1960	—	—	—	○	—	—	◎	◎
1961	—	◎ A	—	○	● A	◎	—	◎
1962	—	○	—	◎	—	○	○	—
1963	—	—	—	—	—	○	—	○
1964	—	—	○	—	—	○	—	○
1965	—	—	○	—	—	○	—	—
1966	—	—	○ B	—	—	○	—	—
1967	—	—	—	—	—	○	○ A, E	—
1968	—	—	—	—	—	—	—	—
1969	—	○	—	—	—	—	—	○ D
1970	—	—	—	—	—	—	◎	○ D
1971	—	—	○ E	—	—	○	◎	—
1972	—	—	○ E	—	—	—	◎ D	—
1973	—	—	—	—	—	—	—	—

Note 1. Mortality; □ 10~20%, ○ 20~50%, ◎ 50~70%, ● 70%<
Note 2. Estimated cause of disease; A—Physiological impediment due to high temperature or high salinity. B—Low salinity. C—Pathogenitic micro organisms. D—Parasite (Polydora, Hydroides). E—Unfavorable water quality.

temperature to 21° or 22°C, and the higher temperature the more deaths. High salinities sometime affect mortality rates, but the peak of mass death does not coincide with the temperature of salinity maximum.

6) The distance from the shore and the depth of oyster beds seem to bear no relation to mortality rates.
7) Mass-death occurs neither explosively nor intermittently, but takes place gradually during a spawning season.

A recent study revealed that the most important cause of mass-mortality is the physiological impediment of high water temperature during spawning season. This trend is mainly observed on the Pacific Ocean side of Japan.

In the case of the Japan Sea, the causative agent of mass-death is simply explained by temperature and salinity change, though physiological impediment is observed.

Generally, mass-death occurs more frequently in the southern part than in the northern part of Japan, and also is higher on the coast of Pacific Ocean than on the coast of the Japan Sea (Table 1).

Mass-mortality has appeared since 1945 and is considered to be coincident with the development of hanging method and the increase of productivity at each culture ground. Moreover, recent culture techniques seek earlier fattening, harvest and higher production. As a result, the trend of mass-mortality is

Table 2. Change of oyster culture in Japan

	Year	Production (Meat) t	Total No. of Growers	Raft		Long Line		Simple-Hanging		Stick		Bottom	
				No. of Growers	No. of Raft	No. of Growers	No. of Long-Line	No. of Growers	Area (10^3m^2)	No. of Growers	Area (10^3m^2)	No. of Growers	Area (10^3m^2)
Total	1961	23,352	8,294	3,645	10,790	890	3,891	2,986	2,272	440	961	1,167	8,491
	1971	27,865	5,536	2,911	14,356	1,204	10,615	1,299	3,854	60	316	323	2,098
	71/61	1.19	0.67	0.80	1.33	1.35	2.73	0.44	1.70	0.14	0 33	0.28	0.25
Hokkaido-Region	1961	1,115	298	6	8	39	31	151	186	—	—	102	923
	1971	298	340	2	2	188	4,124	—	—	—	—	150	1,057
	71/61	0.27	1.14	0.33	0.25	4.82	133.0	—	—	—	—	1.47	1.15
Pacific Side North-Region	1961	5,745	4,444	2,247	2,771	851	3,860	1,367	277	—	—	271	1,633
	1971	4,439	2,923	1,369	1,957	1,007	6,458	618	137	—	—	77	32
	71/61	0.77	0.88	0.61	0.71	1.18	1.67	0.45	0.49	—	—	0.28	0.02
Middle-Region	1961	368	447	153	129	—	—	221	371	72	282	—	—
	1971	606	327	181	336	—	—	146	1,433	—	—	—	—
	71/61	1.65	0.73	1.18	2.60	—	—	0.66	3.86	—	—	—	—
South-Region	1961	47	71	62	59	—	—	9	13	—	—	—	—
	1971	4	62	62	26	—	—	—	—	—	—	—	—
	71/61	0.17	0.87	1.00	0.44	—	—	—	—	—	—	—	—
Japan Sea Side North-Region	1961	938	308	193	560	—	—	173	255	—	—	—	—
	1971	847	272	179	601	—	22	90	229	—	—	—	—
	71/61	0.90	0.88	0.93	1.07	—	—	0.52	0.90	—	—	—	—
West-Region	1961	458	456	232	277	—	—	230	75	—	—	—	—
	1971	98	150	79	81	—	—	71	165	—	—	—	—
	71/61	0.21	0.33	0.34	0.29	—	—	0.31	2.20	—	—	—	—
East China Sea-Region	1961	1,517	858	125	38	—	—	17	4	248	550	666	5,722
	1971	264	166	69	94	—	—	—	—	50	280	47	882
	71/61	0.17	0 19	0.55	2.47	—	—	—	—	0.20	0.51	0.07	0.15
Seto-Inland Sea-Region	1961	13,148	1,412	627	6,948	—	—	818	1,080	120	128	128	209
	1971	21,310	1,296	970	11,259	—	11	374	1,890	10	36	49	127
	71/61	1.62	0.92	1.55	1.62	—	—	0.46	1.75	0.08	0.28	0.38	0.61

spacially expanding. The cause of mass-mortality is also confused by increasing eutrophication in coastal waters.

THE PRESENT STUDY OF MASS-MORTALITY

We define mass-mortality as the death of 50–60% of the oysters. This is boundary of existance of oyster culture. Mortality rates of more than 60% means the death of commercial oyster culture.

The present study of mass-death has two main purposes. The first is to identify the causative agent of mass death. The other is to find methods for preventing mass-death in the hanging system of oyster culture.

Mass-death of oysters has occurred every year since 1961 in Matsushima Bay, Miyagi Prefecture. It has taken place since 1969 in several important oyster culture beds; particularly in the Western region. Mass-mortality rates in both areas have reached at 40–60% annually.

However, the causative agents in the mass-death of oyster in Japan have never been clarified, though it was assumed to be due to unfavorable environmental condition, certain infections pathogens, parasites and fouling organisms.

In this paper, I summarize the status of these studies as follows: Many workers have carried out ecological, physiological and pathological studies attempting to determine the causative agent of the mass-death in Matsushima Bay and Hiroshima Prefecture. Both areas are the main oyster culture grounds on the Pacific and are exposed to advancing eutrophication. Moreover, the culture techniques in these areas are highly developed and the majority of oyster growers have three or four kind of culture grounds; namely seelding ground, hardening ground, growing ground and fattening ground. Each ground is used for a special purpose to achieve earlier fattening and harvest.

As I have shown previously, mass-mortality usually occurs in spawning period in Japan. In a recent study, the following biological results were obtained:

1) The rapid sexual maturation and the mass spawning cause a heavy physiological involution, which finally results mass death.

2) Bacteria invade through the mouth and move to various parts of the body through the blood vessels and the spaces between the glycogen-bearing cells.

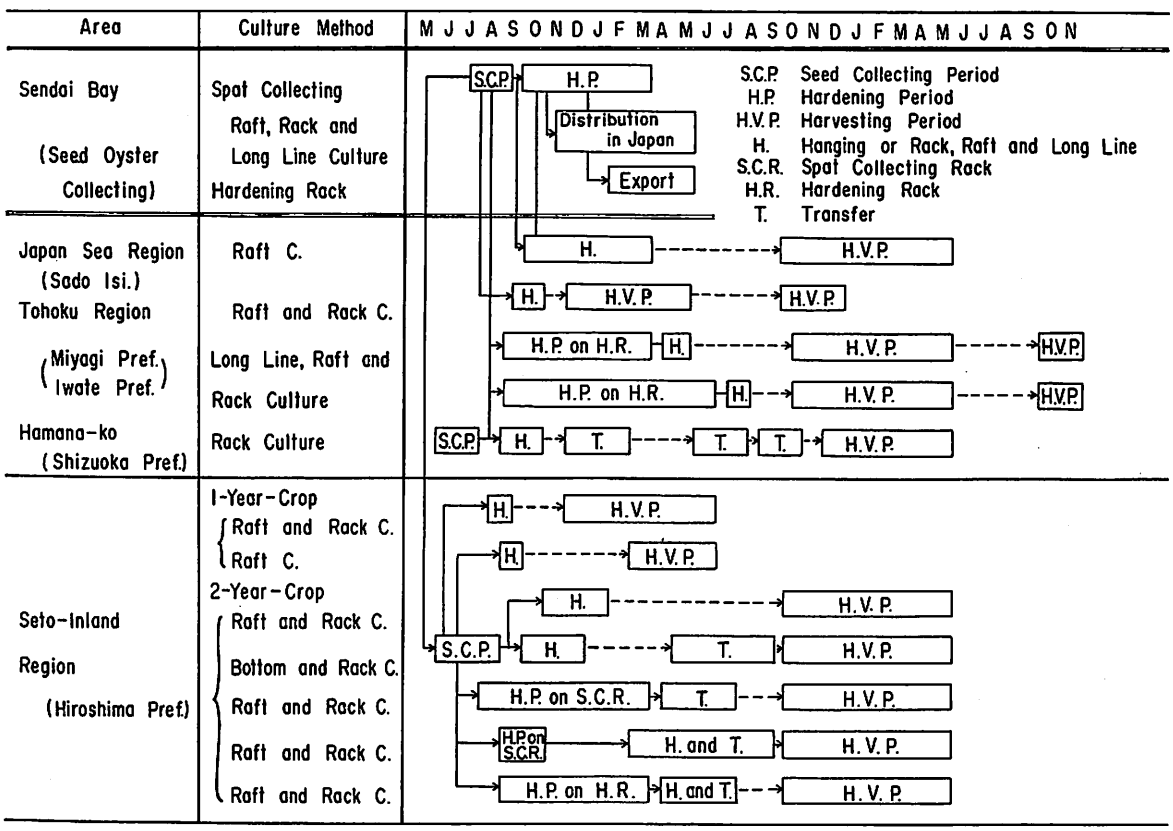

Figure 2. Seasonal activity of oyster culture in Japan.

No definite coincidence is observed between the bacterial infection and the oyster death. Such behaviour of bacteria seems to indicate that bacterial invasions occur only after oysters suffer from the physiological involution resulting from rapid sexual maturation and mass spawning.

3) Serious pathological changes such as enteritis and multiple abscesses, are observed occasionally during the spawning season. These serious changes are rather rare and are not regarded as the causative agent of mass-death.

At the present time, many workers are of the opinion that the causative agent of the mass-death of oyster is most likely due to the physiological disorder and metabolic disturbance derived by heavy gonad formation and massive spawning under high water temperature and eutrophication of the environment. Mass-death is not considered to be primarily due to the infection by bacteria and/or amoeboid parasites.

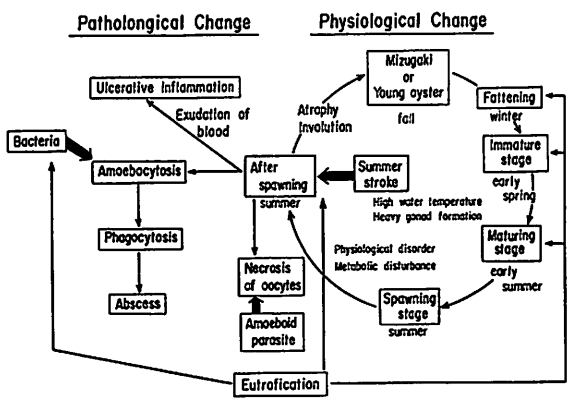

Figure 3. Diagramatic illustration of the relation between physiological cycle and pathological changes in cultured oyster in Japan.

Oyster growers are adopting two growing methods to prevent heavy gonad formation and massive spawning. In the first, hardened seeds are used to prevent the heavy gonad formation. In the second, oyster are placed in poor nutritional areas during the gonad-maturing period, and are moved to nutritionally rich places in late summer where they fatten during fall and winter. Both methods prevent and postpone gonad formation. The use of hardened seeds and the transplantation will result not only the economization of labour and other expense but also low mortality and better quality.

CONCLUSION

The oyster culuture in Japan holds a historical honor, for it began before the science in this field.

The oyster mass-mortality arose in 1945 when the hanging culture technique was induced. The hanging culture has the advantage of three dimensional use of the sea, and enables groweres to expand the farm to deeper areas beyond restricted shore areas. As the result of adoption of hanging technique, oyster production increased rapidly.

The mass-mortality arose in close relation with the successive improvement of culture techniques, which brought about rapid fattening, growth and earlier harvest. It is primarily caused by physiological disorder and metabolic disturbance which result from extraordinary maturation of gonads under the nutritionally rich and high temperature conditions.

Seed oyster produced in Sendai Bay are famous worldwide and fulfill not only the dometic demands but also those of the United States and France. Therefore, the mass-mortality of Pacific oysters is a problem not only in Japan but in wider areas. With more extensive oyster production in future, more problem will need to be solved. Greater advancement in solving these problems can be expected with the international cooperation of experts in this field.

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MARINE DISEASE PROBLEMS IN THE UNITED STATES

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ABSTRACT

Marine diseases have received increasing attention recently because of widespread epizootics and mortalities in natural populations of animals, because of the severe limiting effect of disease in marine aquaculture, and because of the possible relationships of coastal pollution with disease. The important role of stress as a determinant of disease is receiving increased attention, as are the internal defense mechanisms of marine animals. Control of marine diseases is severely limited, even in mariculture environments.

INTRODUCTION

Economically successful aquaculture in the United States is largely limited to a few fresh-water or anadromous species—trout, salmon and catfish. Of the marine animals, only oysters and clams form the basis for successful mariculture industries. For a number of other marine species—such as shrimps, lobsters, crabs, scallops, pompano—we are still in what can be described as the experimental or pilot plant stage.

One of the urgent requirements of the moment is for a more adequate technological base for mariculture. Such a base, including such areas as complete diets, genetically selected stocks, and disease control, is being developed. In the interim period, large investors will wait until such a base is developed, while small entrepreneurs will continue to enter and then disappear after a few years.

Concerning only one part of the technological base —disease diagnosis and control—we are in the phase of describing mariculture diseases as they appear, and attempting to treat them. This process will probably take many years, before we have a complete catalog of diseases and methodology for their control. Even at that point, we will probably be surprised occasionally by new disease entities in cultivated populations. Some indication of the rapidity with which the descriptive phase is progressing in the United States can be seen in the fact that since the initial distribution of a draft handbook of mariculture diseases in the United States in January 1974 (which included description of 42 diseases) an additional 12 diseases have been included in the intervening 8 months—so the printed version of the handbook, scheduled to appear in December, 1974, will contain summaries of 54 disease entities. An equal or greater list of diseases could undoubtedly be assembled from the Japanese literature, which has dealt with diseases of cultivated marine animals for several decades.

This paper will consider four general categories of diseases in marine aquaculture:

(1) diseases and mortalities caused by what can be called "primary pathogens";

(2) diseases caused by facultative pathogens—usually operative in populations stressed by marginal or poor culture environments;

(3) nutritional or stress-induced diseases which are non-infectious (but which may render affected individuals more susceptible to infections); and

(4) the special category of marine animal diseases that may affect humans, and hence can be of actual or potential public health significance.

Additionally, brief consideration will be given to larval diseases, disease resistance, pollution-influenced diseases, and disease control. Throughout the paper, the term disease is used in its broadest possible definition—as any departure from normal structure or function of the animal.

DISEASES CAUSED BY "PRIMARY PATHOGENS"

A few mariculture diseases are caused by primary pathogens—infectious organisms which can act even when culture conditions are reasonably adequate. Included are an extremely virulent bacterial pathogen, *Aerococcus viridans*, which causes "gaffkaemia" of lobsters (Stewart and Rabin, 1970); the haplosporidan protozoan *Minchinia nelsoni*, which caused widespread oyster mortalities on the U. S. Atlantic coast from 1957–1966 (Farley, 1968); an amoeba, *Paramoeba*

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perniciosa, considered to be the cause of mass mortalities of blue crabs on the U.S. Atlantic coast during the period 1965–1971 (Newman and Ward, 1973; and the fungus, *Ichthyophonus hoferi*, a pathogen of many marine fishes (Sindermann, 1970). Other examples could be given, but the important point is that these pathogens are operative in natural populations of marine animals, sometimes at epizootic levels, and they may present problems to marine aquaculture.

DISEASES CAUSED BY FACULTATIVE PATHOGENS

A much larger group of pathogens of cultivated marine animals are those facultative organisms which attack weakened or stressed individuals. Culture conditions such as poor water quality, crowding, inadequate diets, or abnormal water temperatures enhance the effects of such pathogens, which are often normal inhabitants of the environment or the animals. Included in this category are vibrios, pseudomonads, aeromonads, pasteurellas, and possibly some of the recently described viruses of marine invertebrates (Couch, 1974; Farley, et al., 1972). The vibrios have been of particular concern to United States aquaculture efforts in recent years, and this bacterial group at present constitutes one of the most significant deterrents to successful culture of several species. Vibrio disease occurs in cage-reared salmon on the Pacific coast (Cisar and Fryer, 1968; Evelyn, 1971); vibrio infections produce mass mortalities of shrimp larvae and juveniles in the Gulf states (Lightner and Lewis, in press), and vibrios have been implicated in fin rot disease of a number of fish species (Mahoney, et al., 1973; Sindermann, in press).

An excellent summary of our knowledge of the relationship of environmental stress and fish diseases has just been published (Snieszko, 1974).

NUTRITIONAL, GENETIC, OR STRESS-INDUCED NON-INFECTIOUS DISEASES

A number of abnormalities in cultivated marine fish and shellfish, which fall within a broad definition of disease, can be traced to a non-infectious origin. In some instances, inadequate nutrition is clearly the cause — as in the case of fatty liver degeneration, often accompanied by dropsy, which is found in cultivated fish on incomplete diets (Finucane, 1970). In other instances, the cause is less clear, but results from abnormal embryonic or larval development — as is seen in pug-headedness or vertebral anomalies in fish (Hickey, 1972). In still other instances, the abnormality seems related to severity of environmental stress — as in the case of "spontaneous" muscle necrosis in cultivated shrimps (Rigdon and Baxter, 1970). The affected animals are less likely to survive, and may be more vulnerable to secondary microbial invaders.

DISEASES OF MARINE ANIMALS WHICH MAY BE TRANSMITTED TO HUMANS

Among the many microbial and helminth parasites which infect marine fish and shellfish, only a few have been demonstrated to be pathogenic to humans (Sindermann, 1970b). Microbial diseases of concern include that caused by *Vibrio parahemolyticus*, which has been reported from fish and shellfish, and which causes gastrointestinal problems in humans. Recent outbreaks in humans in the United States have been attributed to consumption of improperly processed shellfish (Nickelson and Vanderzant, 1971; Dadisman, et al., 1973).

Among the worm parasites, a number of heterophyid trematodes from marine and estuarine fishes are capable of infecting humans. Similarly, several larval tapeworms from fish flesh may infect humans. Notable in this respect is *Diphyllobothrium pacificum* which is a common infection in some coastal areas of South America where raw marine fish are eaten. Larval nematodes (Anisakinae) from marine fish can cause severe enteric disturbances in humans. Several cases have been diagnosed in the United States during the past few years. Anisakiasis has been the object of intensive study in Japan and Europe, and several European countries now require freezing of fish such as herring, which carry the larval worms.

It is important to distinguish the above diseases from many other human infections (such as hepatitis and cholera) which may be transmitted passively by shellfish, but which cause no recognizable disease in the marine animals. Such diseases of public health significance must be of major concern in mariculture growout areas for shellfish (oyster, mussels, clams).

POLLUTION-ASSOCIATED MARINE DISEASE PROBLEMS

A matter of particular concern at present in the United States (as it is in Japan and Europe) is the possible effect of environmental contamination on diseases and abnormalities of fish and shellfish. Effects of pollutants may take a variety of forms — most of which fit our broad definition of disease. Increased organic loading of coastal waters by domestic wastes can produce increases in marine bacterial populations, some of which may be facultatively pathogenic to fish, especially if the fish are under stress from industrial chemical contamination. Fin rot disease, which has been reported from a number of marine areas in the United States and in Europe, is an excellent

example of such an association (Sindermann, in press).

Another interesting relationship of disease to pollution concerns the apparent increase in certain microbial infections in fish and shellfish from heated effluents of electric generating stations. Several diseases, including a virus disease of oysters (Farley et al., 1972) and lymphocystis disease of fishes, seem to be enhanced in populations held in such artificial temperature regimes.

Developmental anomalies, such as spinal fusions in fish, and a "stress syndrome" in clams, have been attributed to effects of environmental pollution, even though the association is still somewhat circumstantial. A tenuous association of pollution and increased occurrence of tumors in fish has also been proposed, but much additional data must be assembled before a definitive statement can be made.

Effects of chlorinated hydrocarbons, heavy metals, and petroleum residues on fish and shellfish have been well documented. Larvae are particularly vulnerable effects include mortalities due to acute toxicity, growth retardation, and inhibition of spawning due to chronic exposures.

LARVAL DISEASES

Although fish and shellfish larvae are subject to many of the same pathogens which affect juvenile and adult animals, it is worth-while to focus particular attention on larval diseases. Normally, we lose sight of individual larvae, and think only of the larval population as a whole—probably since any treatment of larval diseases must be mass rather than individual treatment. It has been expressed recently by at least some of those developing commercial mariculture, that the best solution to larval disease problems is to discard the entire culture, since so little time and money has been invested in that culture, as compared to animals approaching market size. This negative approach to larval disease control tends to overlook two critical points:

(1) Sublethal abnormalities and disabilities, both physiological and morphological, may be produced by disease in larval stages, and may be carried through to juvenile and adult stages—often resulting in less than optimum growth rates and a less desirable product;

(2) uncertainty about larval availability at a particular time can dislocate the entire mariculture production system.

Diseases of larvae are often directly related to water quality and nutrition, so effective prophylactic measures include sterilization of water, careful attention to pH and salinity, and proper feeding levels. Molluscan larvae are subject to vibrio and other bacterial infections, which may produce mass mortalities in only a few hours from onset of disease signs. Crustacean larvae are also affected by bacterial diseases, but fungi also seem to be very important, judging from recent reports from shrimp and lobster mariculture efforts. Fish larvae are attacked by a wide variety of microbial and parasitic organisms; even very low levels of parasitism have been shown to have drastic effects on larval survival.

DISEASE RESISTANCE IN MARINE ANIMALS

An important aspect of disease research, both for fish and shell fish, is that of defense mechanisms of the animal. The immunological responses of fish generally parallel those of terrestrial vertebrates, including phagocytosis and the elaboration of specific immunoglobulin antibodies. This fact has been used in attempts to immunize salt-water held salmon against vibrio and other bacterial infections (Fryer et al., 1972; Cisar and Fryer, 1974). High antibody titers can be obtained by intraperitoneal injection of killed bacteria, and some protection of experimental animals is afforded by oral immunization.

Among the invertebrates, response to infection seems to be less specific and largely cellular, although recent work with American lobsters by Paterson and Stewart (1974) has demonstrated increased phagocytic activity, with a suggestion that vaccines prepared from avirulent pathogens (*Pediococcus homari*) produced a limited degree of resistance to gaffkaemia. In other studies, increased bactericidal activity following injection of bacterial antigens was found to exist in lobsters (Acton et al., 1969).

Development of disease resistance in populations of oysters exposed to epizootic levels of particular pathogens is suggested by recent observations. A high degree of natural selection for disease resistance in oyster populations exposed to the protozoan *Minchinia nelsoni* was reported by Andrews and Frierman (1974). An unknown pathogen responsible for extensive oyster mortalities in the Gulf of Saint Lawrence, Canada, has also resulted in selection for disease resistance, in that oysters from other areas die when introduced. The disease is known as "Malpeque Bay Disease".

MARINE DISEASE CONTROL

Preparation of handbook on mariculture disease diagnosis and control (referred to earlier) has disclosed the incompleteness of technology in the general areas of prophylaxis and treatment. Some of the remedies developed for control of fresh-water fish diseases can be adapted for use with marine fish, but the whole

field of invertebrate disease control requires extensive experimentation. Prophylaxis still must emphasize water quality and adequate nutrition. Treatment of diseases includes use of antibiotics—with limited success at high cost—and the experimental use of new drugs (nitrofurans) developed in Japan. Potential difficulties in clearance and approval by the Food and Drug Administration of chemicals used in cultivating sea animals have not yet been faced.

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PARASITES AFFECTING THE PEARL INDUSTRY IN JAPAN

SABURO MIZUMOTO[1]

Parasites of the pearl oyster, *Pinctada fucata*, have been known since the beginning of the pearl industry in Japan, but they did not cause serious damage to the industry in the early years. Only predators such as black porgy, globe fish, eel and octopus were important then. Damage to the pearl industry by parasites has been of particular significance since 1952. The rapid spread of damage over the whole pearl oyster producing area seemed to be caused by the transfer of the pearl oyster from one area to another, in order to complete pearl formation in selected waters or to overwinter oysters in southern, warm waters. Also, lack of biological knowledge and inadequate control measures for the parasites contributed to the widespread damage.

At present, it is well known that parasites and commensals such as *Polydora, Demospongia, Trematoda* and *Malacostraca* affect production of superior mother pearl oysters and pearl production itself. *Polydora* and *Trematoda* cause particularly severe effects on the industry. Prevention and control must always be a consideration.

POLYDORA

The mud worm, *Polydora*, is a well-known organism which produces a mud tube and a blister in bivalve shells. Of the several species of *Polydora* found in shells of the pearl oyster, *Polydora ciliata* is recognized as the most important (Mizumoto, 1964). Damage to the industry by mud worms has occurred since 1960 in the whole pearl oyster area. According to observations in 1970, more than 50 percent of pearl oysters cultivated in the main farm areas — Mie, Wakayama, Ooita, Kumamoto, Miyazaki and Kagoshima — in the central and southern waters of Japan were infected. Oysters were weakened by the adverse conditions and in some cases heavy mortalities resulted.

Polydora ciliata in Ago Bay, Mie, spawn during the period May to October, mainly in May, June, and October. The egg is spherical with a diameter of about 98μ and with a capsule which attaches to the inner wall of the tube. The eggs develop within the capsule and take nine days at a temperature of 21.5°C or seven days at 26.3°C to grow to the larval stage after fertilization. In summer and winter, the larvae settle on the shell surface. In summer settling occurs in a remarkably short time. Mud tubes and blisters of the worm are predominantly found in August and January (Mizumoto, 1964, 1966, and 1968).

To exterminate mud worms, the industry is using brine treatment in the larval setting season. The treatment is as follows: pearl oysters, previously removed from hanging culture, are dipped into seawater for five minutes, next in fresh water about 15 minutes, then in 22 percent brine for 20 minutes and then dried in the shade for 15 minutes. Biological control, using natural enemies of mud worms, is also being explored.

TREMATODA

A disease of pearl oysters caused by the trematode, *Bucephalus varicus*, seriously affects the pearl industry. Pearl oysters infected by worms cannot be used in any pearl production, neither as mother shell nor as piece shell, because they produce only poor pearls with spotted or thin pearl layers. Formerly, the disease was found in limited areas—Gokasho, Tanabe, and Hirajo—in the central coastal area of Japan. As the industry developed, the disease has spread over the whole pearl oyster producing area. In some pearl farms, 30 to 40 percent of the pearl oysters have been infected.

In 1934, the bucephalid trematode parasite of pearl oysters was studied and described by Ozaki and Ishibashi as *Bucephalus margaritae* n. sp., but Sakaguchi (1968) proved recently that the disease was caused by larvae of *B. varicus* which is the same as *B. margaritae*. He also completed the life cycle of the worm. Larval trematodes found in pearl oysters, as the first intermediate host, are young sporocysts. The young sporocysts become mature in spring when the water

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temperature rises.

The germ-balls in mature sporocysts become young cercaria. Through summer and autumn the cercaria mature and leave the pearl oyster host. They invade the muscular tissue of small fishes—*Atherina bleekeri, Spratelloides japonicus,* and *Engraulis japonica*— as the second intermediate host, and become metacercariae. These fishes are abundant in the water near the pearl oyster farms. Final hosts of the trematode are large carangid fishes, *Caranx sexfasciatus, C. equula,* and *C. ignobilis.* The metacercariae grow to adults in the digestive organs of the *Caranx* (Sakaguchi, 1968).

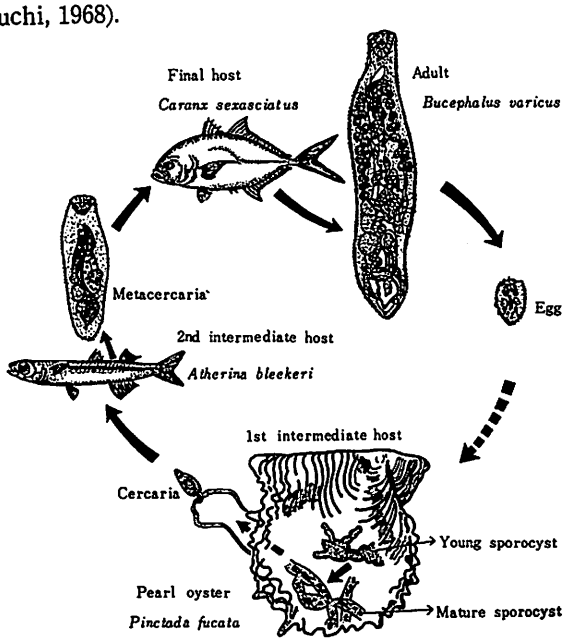

Figure 1. The life history of the trematode parasite in pearl oyster.

The only practical control of the parasite is to transfer the pearl oysters from infected to non-infected waters in September, the month when trematode invasion normally takes place. It is impossible to remove the host fishes from the coastal areas.

Another trematode, *Proctoeces ostrea,* is found in the internal organs, mainly heart and gonad, of the pearl oyster, and the parasite is common throughout the whole pearl oyster producing area. The damage caused by *Proctoeces* is not severe at present, but it may become an important industry problem in the future (Sakaguchi, *et al.*, 1970 ab: Sakaguchi, 1970).

DEMOSPONGIA AND MALACOSTRA

Damage caused by the boring sponge, *Cliona* sp., is minor in the industry. Oyster crabs, *Pinnotheres* sp., were reported as harmful to the industry in some cases. In Ikeda Bay, Kagawa, in 1965, about 50 percent of pearl oysters were parasitized by crabs.

The author wishes to thank Dr. Hisashi Kan-no, Tohoku Regional Fisheries Research Laboratory, Dr. Koji Wada and the staff of the Pearl Research Laboratory for their help in carrying out this study.

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FUNGAL DISEASES OF MARINE CRUSTACEA

CHARLES E. BLAND, Ph. D.[1]

Of the more than 50,000 species of fungi known to man, it is surprising to note that only 450–500 have been described as marine. Even more surprising is the fact that of these 450–500 species of marine fungi, less than 25 have been described as pathogens of marine animals. Table 1, which was prepared (Bland, 1974) for a recent symposium on "Diseases of Aquatic Animals in the Gulf Coast Area" lists some of the better known fungal parasites of marine animals and gives their probable taxonomic placement within the major groups of fungi. (For information relative to these and other fungal diseases of marine organisms,

Table 1. Major Fungal Pathogens of Marine Animals

Major taxonmic group	Total number of marine taxa	Pathogenic genera	Hosts
Phycomycetes	50 (approx.)	*Ichthyosporidium hoferi* (Plehn & Mulsow) Pettit* (*Ichythophonus hoferi* Plehn & Mulsow*)	A variety of both marine and freshwater fishes
		Atkinsiella dubia (Atkins) Vishniac *Plectospira dubia* Atkins	Ova & larvae of the pea crab and several other marine invertebrates
		Haliphthoros milfordensis Vishniac	Ova & embryos of *Urosalpinx cinerea* & ova of *Pinotheres pisum*
		Pythium thalassium Atkins	Ova of the pea crab & other invertebrates
		Pythium spp.	Shrimp
		Saprolegnia parasitica Coker	Larvae of *Palaemonetes kadiakensis* Atlantic salmon?
		Leptolegnia marine Atkins	Ova & larvae of pea crab
		Spongiophaga communis Carter*	Various sponges
		Sirolpidium zoophthorum Vishniac	Larvae and juveniles of oysters and clams
		Lagenidium callinectes Couch	Ova & larvae of crabs, shrimp, and barnacles
		Lagenidium sp.	Internal organs of *Lampanyctus ritteri*
Ascomycetes	200 genera with over 300–400 species One-third are obligately marine	*Trichosporon ladderi*	Implicated in shrimp disease
Fungi inperfecti	50+	*Fusarium*	American lobster and shrimp
Basidiomycetes	3	Unknown	
Organisms of uncertain Affinities Labyrinthulales	12 (approx.)	*Labyrinthomyxa marina* Mackin & Ray* (*Dermocystidium marinum* Mackin, Owen and Collier)*	Bivalve molluscs

*Taxonomic placement uncertain

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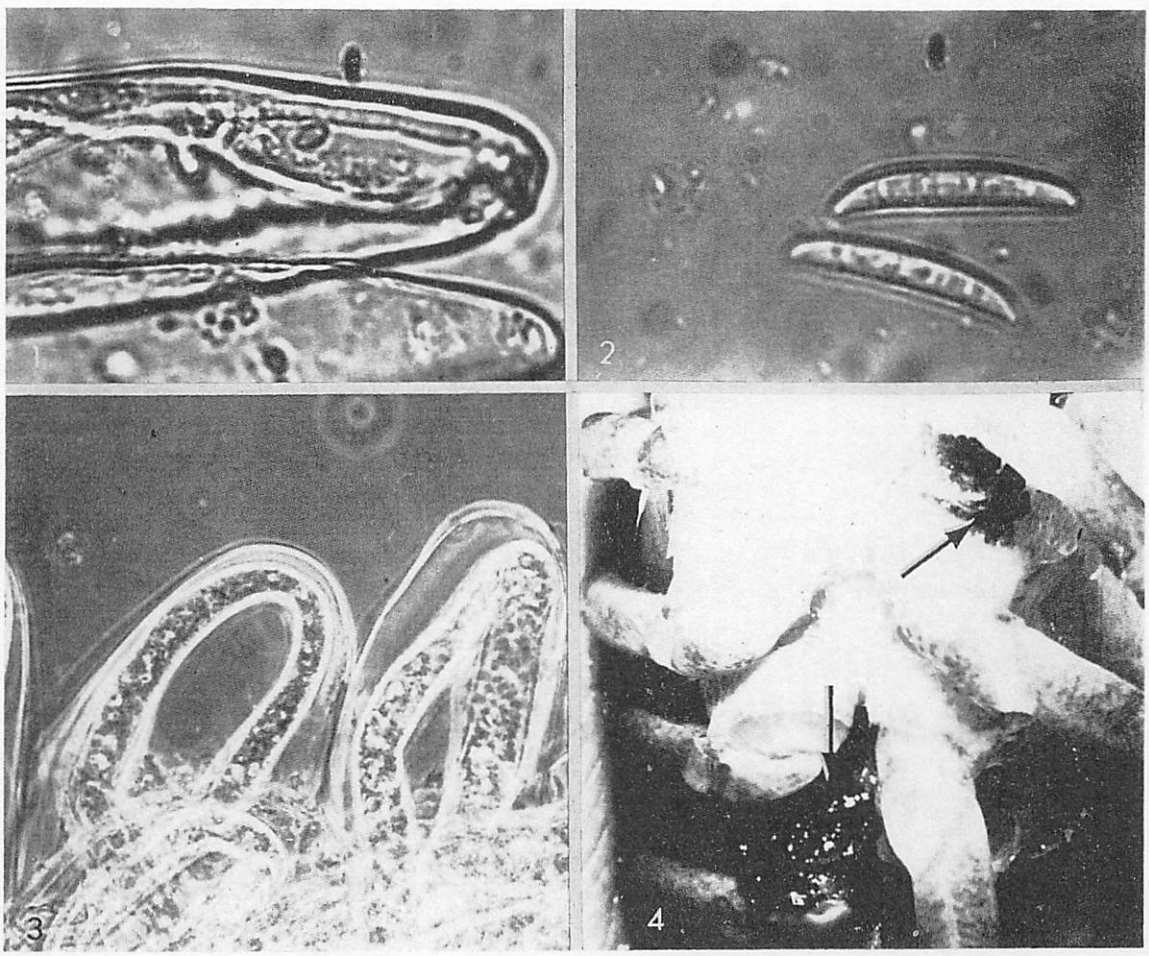
readers are referred to references as follow: Johnson and Sparrow, 1961; Snieszko, 1970; Sindermann, 1970, 1974). With only a brief examination of this chart, it is easy to see that with only two rather important exceptions, *Ichthyosporidium hoferi* parasitic on the muscle and internal organs of both marine and freshwater fishes, and *Labyrinthomyxa marina*, parasitic on a variety of bivalve molluscs, a majority of the other known parasitic marine fungi occur primarily on ova and larvae of marine crustacea. This is important in view of the fact that the aquaculture of several commercially important marine crustacea is being attempted at a number of laboratories and field stations throughout the world. With this in mind, therefore, I would like first to familiarize you with some of the fungi which have already caused problems in the culture of marine crustacea and finally to review briefly the efforts going on in our laboratory toward, hopefully, the gaining of solutions to these problems.

Of those fungi listed in Table 1, only three thus far have proved to be serious problems in crustacean aquaculture. These include two phycomycetous genera, *Haliphthoros* and *Lagenidium* and one member of the Fungi Imperfecti, *Fusarium*.

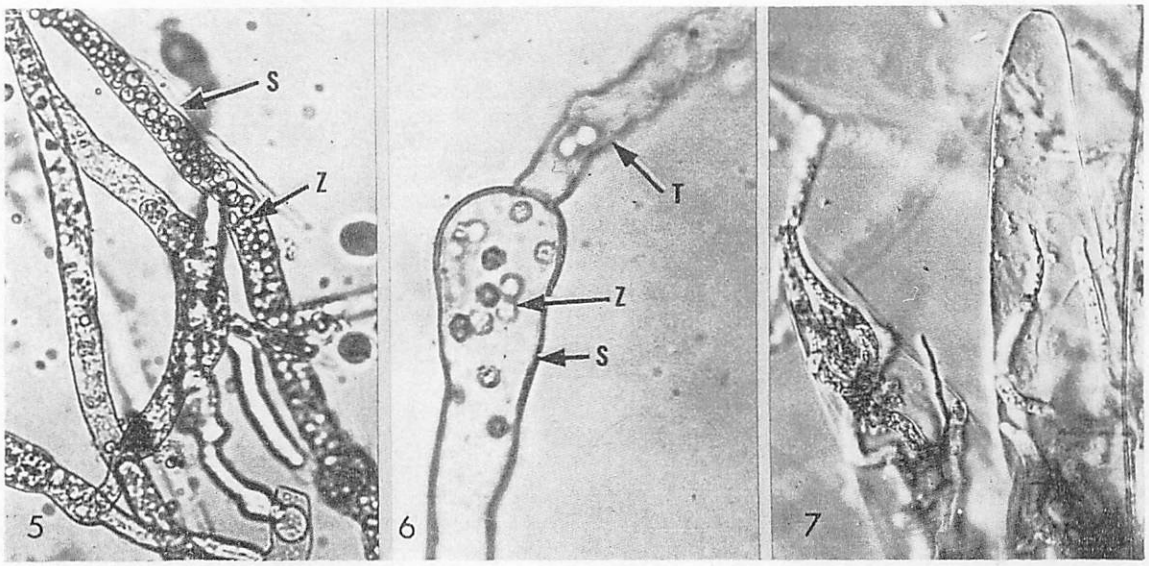
FUSARIUM

In a recent article, Johnson (1974) of the Fish Disease Diagnostic Laboratory, Texas A & M University, College Station, Texas, reported infection of laboratory held pink shrimp (*Penaeus dourarum*) with the imperfect fungus, *Fusarium*. Beginning development primarily in the gills and antennal scales (Figs. 1 & 2), hyphae of the fungus were reported to develop over a two week period into more than 10% of the body area. Incidence of infection was restricted to less than 5% of the shrimp held in the laboratory aquaria.



Figures 1 and 2. *Fusarium sp.* on gills of *Penaeus dourarum*. Fig. 1. Hyphae inside gills. Fig. 2. Conidia. (Photos courtesy of S. K. Johnson).

Figures 3 and 4. *Haliphthoros* sp. on *Homarus americanus*. Fig. 3. Hyphae inside gills of juvenile lobster. Fig. 4. Scab at site of infection on ventral side of juvenile (arrows). (Photos courtesy of W. S. Fisher).



Figures 5–7. *Haliphthoros* sp. from *Penaeus dourarum*. Fig. 5. Sporangium (S) with zoospores (Z). Fig. 6. Sporangium (S) with zoospores (Z) being discharged through tube (T). Fig. 7. Hyphae inside gills.

Johnson noted that infected shrimp did not develop a black gill condition such as that reported by Equsa and Ueda, (1972) for a *Fusarium* infection of *Penaeus japonicus*. It might further be mentioned that Lightner and Fontaine (personal communication) have observed also *Fusarium* infections of both shrimp and lobster. For further information concerning the latter as well as another reported case of *Fusarium* infection of shrimp, please see the paper by Lightner which is included in this report.

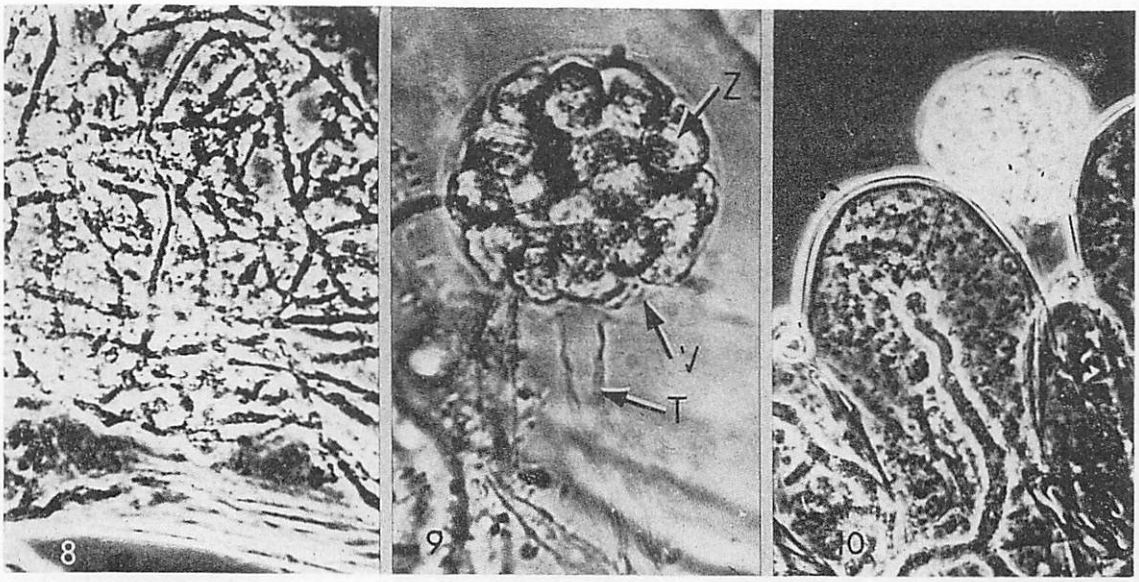
HALIPHTHOROS

Vishniac (1958) first described *Haliphthoros* from ova and embryos of the oyster drill, *Urosalpinx cinerea* and by innoculation, from ova of *Pinnotheres pisum*. Recently, problems concerning *Haliphthoros* have developed at the Bodega Marine Laboratory, Bodega Bay, California, where large colonies of both the lobster *Homarus americanus* and *H. gammarus* are maintained. As reported to us by E. H. Nilson and W. Fisher (personal communication), larvae and young juveniles are both susceptible to infection with *Haliphthoros*. In this disease, the fungus apparently first attacks the gills (Fig. 3). From the gills, the mycelium of the fungus spreads throughout the muscle tissue. Host response to the fungus is by the formation of a dark red scab at the area of heaviest infection (Fig. 4). Death of the host occurs during molting where it appears that the scab and mycelium of the fungus result in a cohesion between the exoskeleton and the sub-skeleton which hinders ecdysis. The destructive potential of *Haliphthoros* is demonstrated in that of 1,000 *H. americanus* larvae contained in closed system aquaria for 22 days, 46% of the larvae died with 95% of the dead animals showing symptoms indicative of infection with *Haliphthoros*.

Evidence that strains of *Haliphthoros* may be of concern to persons engaged in the culture of crustacea other than lobster was obtained this summer at the Duke University Marine Laboratory, Beaufort, N. C. by the author and a graduate student, T. Tharp, when attempts were made to maintain adult specimens of the pink shrimp, *Penaeus duorarum*, in self-contained aquaria. Although initial interest in these shrimp concerned their susceptibility to the fungus *Lagenidium*, it was found that of the over 24 specimens under study, all succumbed within a two week period to infection with *Haliphthoros* (Figs. 5–7). Although initial infection was confined to the gills, it was possible also to isolate this fungus from the eyes of heavily infected individuals. There was no apparent invasion of the fungus into the muscle tissue. This strain of *Haliphthoros* is now under study and is being compared for purposes of taxonomy and possible control with the isolate of *Haliphthoros* from lobster which was kindly provided by Fisher and Nilson, mentioned previously.

LAGENIDIUM

Concerning the fungus *Lagenidium*, Couch (1943) was the first to describe a member of this genus, *Lagenidium callinectes*, as being parasitic on ova of the blue crab, *Callinectes sapidus*. Although originally considered by Rogers-Talbert (1948) to be only a peripheral parasite of the crab egg mass or "sponge" and not likely a serious threat to the reproductive potential of blue crabs, recent evidence indicates that the



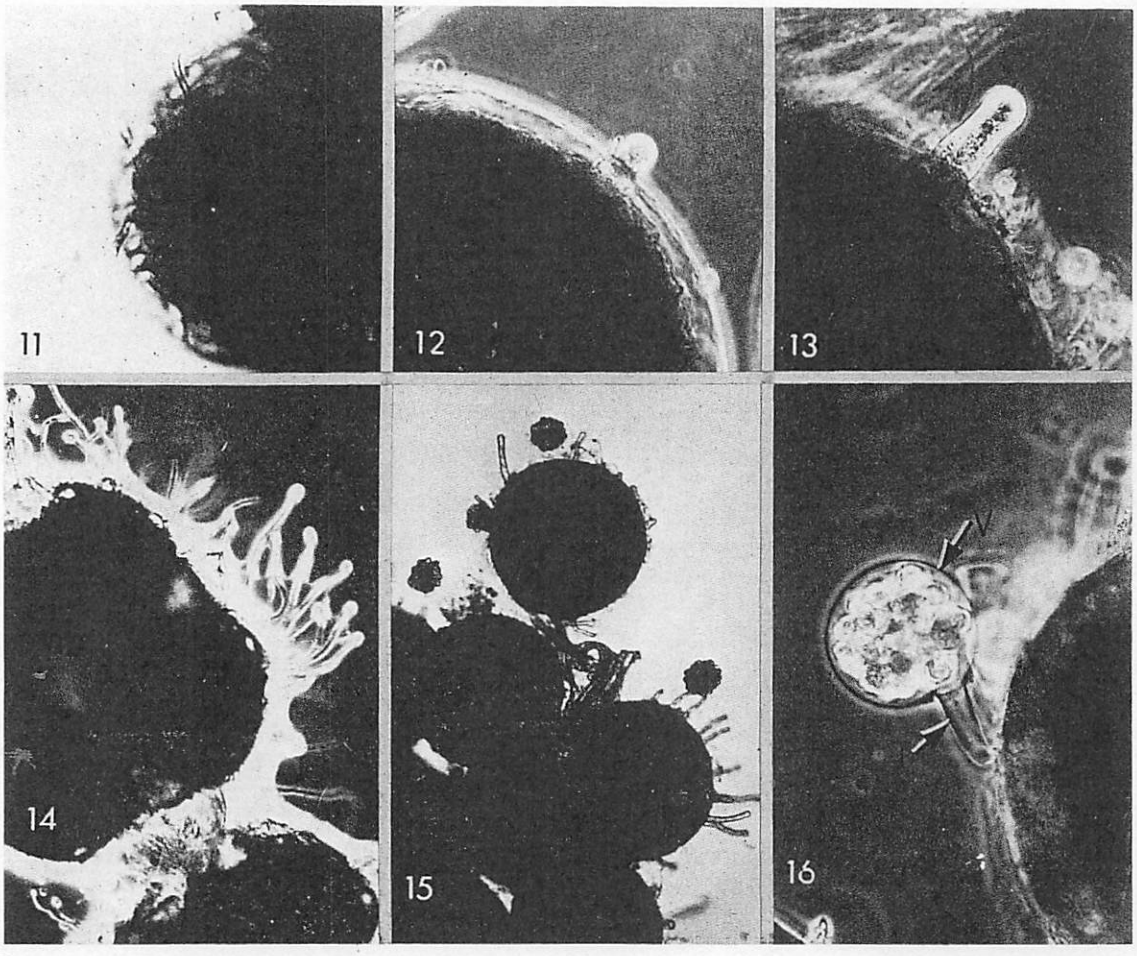
Figures 8–9. *Lagenidium* sp. on *Cancer magister*. Fig. 8. Hyphae inside dorsal spine at juncture of head. Fig. 9. Zoospores (Z) within vesicle (V) at tip of discharge tube (T) photos courtesy of D. Armstrong. Fig. 10. Hyphae within gills of *H. Americanus*. (Photo courtesy of U. S. Fisher).

same or closely related members of the genus *Lagenidium* may pose a severe threat to the successful culture of several commercially important marine crustacea. To the author's knowledge, problems in crustacean aquaculture which were directly attributable to infection with strains of *Lagenidium* have occurred in at least five instances which are as follow:

Investigators at the National Marine Fisheries Service Laboratory in Galveston, Texas, Lightner and Fontaine (1973), described a primary mycosis of the white shrimp, *Penaeus setiferus*, which they attributed to a strain of *Lagenidium*. In infected shrimp, which were in the second protozoeal stage of development, the thorax, abdomen, eye stalks, and even the swimming appendages were eventually filled with hyphae of the fungus. Shrimp mortality reached a level of 12.4% with there being no sign of the disease after the shrimp reached the first mysis stage of development. Sporulation of the fungus, which will be described later for a similar fungus on crab ova, commenced soon after the infected shrimp had become completely immobilized and settled to the bottom of the rearing tank. In an experimentally induced infection of the brown shrimp, *Penaeus aztecus*, with spores of *Lagenidium* these same investigators described a mortality attributable to the fungus of approximately 20%. (For further information concerning this infection of shrimp by *Lagenidium*, readers are referred to the paper by Lightner which is included in this report.)

At the Oregon State University Marine Science Center, Newport, Oregon, Armstrong and Ho have revealed (personal communication) that experiments involving larvae of the Dungeness crab, *Cancer magister*, were interrupted during the summer of 1973 by the sudden death of all of the larvae. After microscopic examination of the diseased larvae, the cause of death was attributed to a filamentous fungus (Fig. 8) which was subsequently identified by its mechanism of spore formation (Fig. 9) as belonging to the genus *Lagenidium*. A paper concerning the general infection of Dungeness crab larvae by this fungus and possible methods of treatment is now in preparation by Armstrong and should be available in the near future.

At the Bodega Marine Laboratory, Bodega Bay, California, in addition to encountering problems relative to *Haliphthoros* infection of larval lobster, Fisher and Nilson (personal communication) report that on two recent occasions they have encountered mortalities of larval lobsters (*Homarus americanus*) which could be attributed to infections by fungi of the genus *Lagenidium*. Although the causative organism which was isolated from larvae after each of the two incidences of infection was clearly *Lagenidium*, Nilson reports that there are obvious differences in size between the two and that the pattern of infection in the lobster larvae is different. In one isolate, the larger, the fungus is never seen beyond the fourth molt and invades only the epidermal layer under the carapace and occasionally also the muscle tissue of the appendages and tail. The smaller isolate, however, is regularly seen in the fourth and fifth stage animals and invades, in addition to the tissues mentioned for the larger form also the midgut gland and the gills (Fig.



Figures 11–16. *Lagenidium* sp. on ova of *Callinectes sapidus*. Fig. 11. Hyphae inside ovum. Figs. 12–13. Growth of hypha through surface of ova. Fig. 14. Growth of hyphae from infected ova into agar medium. Fig. 15–16. Sporulation on ova. Note vesicle (V) at tip of discharge tube (T).

10). A manuscript concerning the nature of this infection of lobster larvae with *Lagenidium* is now in preparation by Nilson and Fisher. For purposes of taxonomy and further study, isolates have been deposited in the author's laboratory in order that comparisons may be made between this and other pathogenic marine strains of *Lagenidium* which are in culture.

In addition to the above, the author is aware also that fungi, apparently of the genus *Lagenidium*, have been implicated in disease problems experienced in other efforts at crustacean aquaculture. First, at the Dow Chemical Company's shrimp hatchery at Freeport, Texas (Cook, 1971; Hysmith, personal communication) extensive mortality of brown shrimp larvae, *Penaeus aztecus*, within 3 to 5 days has been encountered on several occasions and has been attributed to a species of *Lagenidium*. Also, J. A. Barkate of the Ralston Purina Company, St. Louis, Missouri, reported before the meeting of the World Mariculture Society, Charleston, South Carolina (1974) that in their aquaculture efforts they have seen evidence of various fungi in shrimp, which seem to increase mortalities. The fungi illustrated in the lecture presented by Barkate appeared to be of the genus *Lagenidium*.

In the author's laboratory, investigations concerning *Lagenidium* were centered initially around investigations into the destructive potential of this organism to the blue crab, *Callinectes sapidus*, fishery of North Carolina. Toward this goal, studies have now been completed into the occurrence, distribution, life history and cytology of a strain of *Lagenidium* (L-1) which was isolated from ova of the blue crab. References covering publication of the completed portions of these studies are listed in the bibliographic section of this paper (Amerson and Bland, 1973; Bland and Amerson, 1973a, 1973b; Bland and Amerson, 1974) and the results contained therein will not be discussed in detail at this time. However, a brief review of some of our findings relative to *Lagenidium* infection of the

Table 2.

Fungicide	Minimum Lethal Concentration LC_{100} in ppm		Effect on Larval and Adult Invertebrates
	L-1 (isolated from *C. sapidus*)	L-3B (isolated) from *P. setiferus*)	
Benlate, 50% WP	29	39	Not tested
Captan, 50% WP	3.2	5.0	No effect on adult blue crab, lethal to blue crab larvae Lethal to *Penaeus aztecus* at effective dosage[2,3]
Dichlone	10	21	Not tested
Difolatan, 39% Flow	7.2	8.2	Not tested
Dyrene, 50% WP	6.0	21.0	Not tested
DS 9073[1]	1.3	3.2	Not tested
Manzate 200/Dithane M-45 80% WP	2.1	2.9	No effect on adult blue crab, lethal to blue crab larva Lethal to *Penaeus aztecus* at effective dosage[2,3]
Terraclor, 75% WP	1.3	4.3	Studies underway
Tribasic Copper Sulfate	159	150	Not tested
Vitavax, 75% WP	37.5	38.0	Not tested
Malachite Green 99% Pure	0.006	0.01	Studies underway
Treflan, 45% Flow	5.0	3.0	Studies underway

[1] An experimental fungicide/bactericide from ICI America Inc.
[2] Tests conducted by Drs. J. Costlow, D. Lightner. or Mr. B. Hysmith.
[3] When adult species of *Murex, Uca, Arbacia, Astaria, Nassarius, obsoleta,* and *Terebra dislocata* were placed in concentration of Captan and Manzate 200 lethal to *L. callinectes*, no adverse effects were observed during the 5-day exposure.

blue crab and a summary of our current efforts concerning fungal diseases of marine crustacea is in order.

The geographic location of collecting sites and research headquarters has been the coastal area of North Carolina in the vicinity of Morehead City and Beaufort where facilities of the Duke University Marine Laboratory have been utilized. For sampling, crabs, primarily *Callinectes sapidus*, were collected in wire-mesh traps which are referred to locally as "crab pots." In these traps, the crabs are enticed into the cage through openings to centrally placed bait At this point the cage is so constructed as to prevent escape of the crabs. After collection, the crabs are sorted as to sex and ovigerous condition of the female specimens. On return to the laboratory, certain of the crabs are maintained for subsequent experimentation in self-contained aquaria, whereas, ova from all of the ovigerous females are examined microscopically for evidence of fungal infection. In general, infected ova may be recognized by their slightly smaller size and greater opacity than the non-infected ones (Fig. 11). However, a sure sign of infection is the observation of fungal hyphae penetrating through the surface of the egg (Figs. 12 & 13). Such naturally occurring infection varies greatly from year to year and even from month to month within a given year. In order to isolate the fungi for further study, infected ova are placed on an antibiotic containing nutrient agar into which the fungus grows (Fig. 14). At this point, it is a simple matter to excise growing tips of the fungus and transfer them to a second nutrient agar plate where they will continue to grow. From either the cultured fungus or from naturally occurring material it is possible to follow the intriguing process of a sexual reproduction in *Lagenidium*. In this process, the cytoplasm from given "septum delineated" portions of the fungal thallus begins to flow through specialized external hyphae and to collect in vesicles which form at the tip of such hyphae (Figs. 15 & 16). Once all of the cytoplasm has been accumulated within the vesicle, the cytoplasm is cleaved into individual zoospores, the infective agents. On release from the vesicle, the zoospores are capable of swimming to other crab ova and there, through a process of germination, penetrate into and thereby infect the ova. This, therefore, is the complete asexual life cycle of *Lagenidium*. A sexual means of reproduction in this organism is unknown except to say that from our electron microscope studies of this organism, which will not be discussed in full at this time, we know that a sexual process must take place at some time during its life cycle because of our discovery of polycomplexes in the nuclei of encysting zoospores. The presence of these

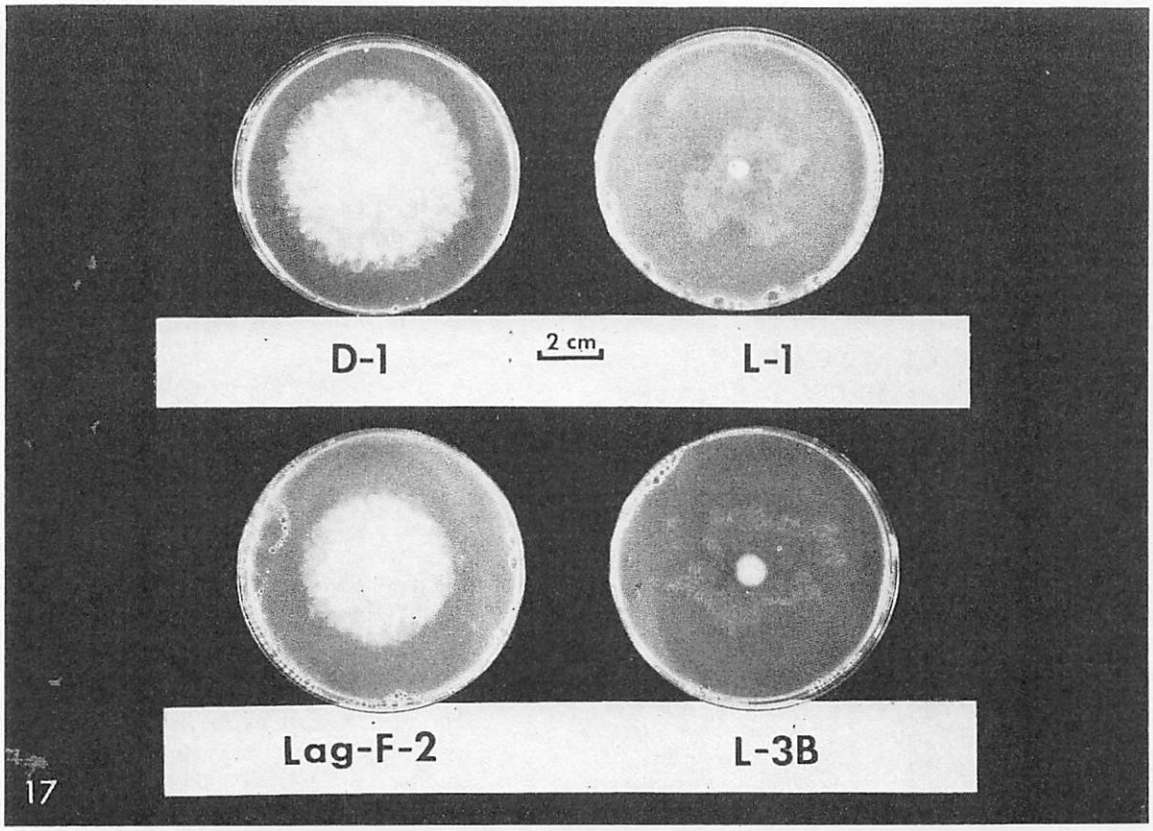


Figure 17. Growth of four differents strains of *Lagenidium* on PYGS agar. (D-1 from *Cancer magister*, L-1 from *Callinectes sapidus*, F-2 from *Homarus americanus*, L-3B from *Penaeus setiferus*).

structures indicates that meiosis has occurred at some prior time and must itself have been preceded by some type of sexual process. Based on this information, therefore, we have been able to propose a complete life cycle for *Lagenidium callinectes*.

At the present time, our main efforts concerning *Lagenidium* and other fungal diseases of marine crustacea are being centered around the following:

1. Studies into the possible chemical and/or natural control of *Lagenidium* and other fungal diseases of marine crustacea when encountered in aquaculture situations. Preliminary results of certain of our tests in this area are shown in Table 2. In addition to the compounds listed in this table, preliminary tests indicate that a natural extract from crab eggs may be highly effective in controlling the spread of *Lagenidium* and possibly other fungal diseases.
2. Further studies into the taxonomy and development of not only *Lagenidium* but also other fungi such as *Haliphthoros* which are of interest because of their possible impact on the future of crustacean aquaculture. The problem which we are facing here is illustrated in the fact that we have observed different rates of growth for all of the strains of *Lagenidium* which we have in culture (Fig. 17). (D-1 from *Callinectes sapidus*, L-3B from *Penaeus setiferus*, D-1 from *Cancer magister*, and Lag F-2 from *Homarus americanus*) all of which at the present time can only be classified as *Lagenidium callinectes*. One need for taxonomic differentiation between these strains is exemplified by the fact that two (L-1 and L-3B) have already been shown to be different in their sensitivity to control measures.

In conclusion, I would like to emphasize firstly the point that it is becoming increasingly evident that pathogenic fungi will have to be of concern to anyone attempting crustacean aquaculture, and secondly the point that there is, at present, a serious shortage of mycologists engaged in the study of fungal diseases of marine crustacea.

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SEASONAL VARIATION IN PHYSIOLOGICAL ACTIVITY OF SCALLOPS UNDER CULTURE IN THE COASTAL WATERS OF SANRIKU DISTRICT, JAPAN, AND A PHYSIOLOGICAL APPROACH OF A POSSIBLE CAUSE OF THEIR MASS MORTALITY

KATSUYOSHI MORI[1]

INTRODUCTION

Scallop fisheries for *Patinopecten yessoensis* in Japan are found mostly from Mutsu Bay and northward. Since 1966, their culture by the hanging method has been extensively carried on not only in these regions, but also in the coastal waters of the Sanriku district which lies to the south of Mutsu Bay (Fig. 1). Its production especially in Iwate Prefecture increased conspicuously with years and reached 3,200 ton in 1971 (fiscal year) and 7,033 ton in 1972, though all of this production does not necessarily mean that the method for culture has been the object of much scientific research. The seeds used for scallop culture in this prefecture have been transferred annually from Mutsu Bay or Hokkaido and the number of them increased from 13 million individuals in 1968 (fiscal year) to 150 million in 1971. This rapid increase was mainly due to the marked expansion of seed production in Mutsu Bay.

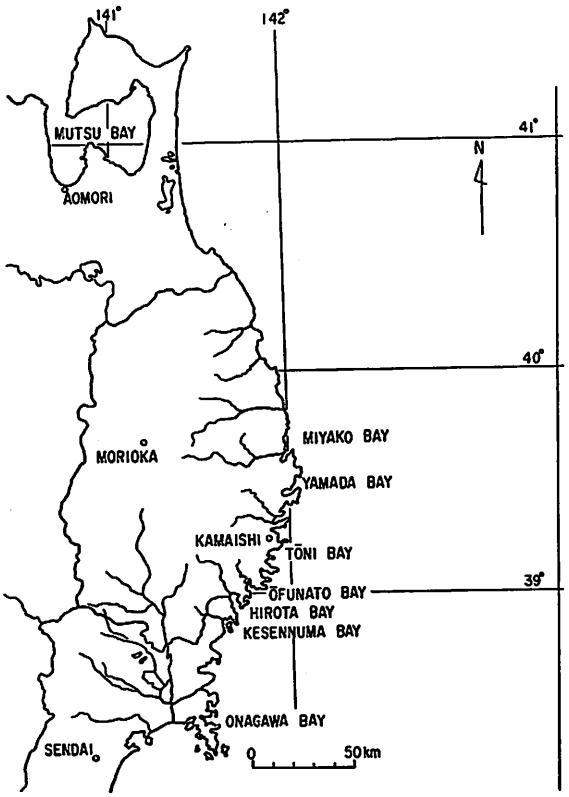

Figure 1. Map of Mutsu Bay and the coastal waters of the Sanriku district.

One of the most troublesome problems that arise when we cultivate the cool-water Japanese scallops, *P. yessoensis*, in the coastal waters of the Sanriku district is that their growth becomes very slow or stops during hot summer, suggesting that high water temperatures may cause the considerable decline in their physiological activity. In 1972 and 1973, the scallop culture in some bays of this region suffered from the mass mortality of more than 50% that occurred from spring to early fall mainly among one year old scallops. Usually, their seeds are transferred to these bays in November or December, so it is suggested that the mass mortality is closely related to the marked decline in their physiological activity caused by unknown factors from the winter to the latter part of the spring. Thus, we strongly feel that the analysis of the seasonal change in the physiological activity of scallop is needed as one of the biological bases for culture management.

SEASONAL VARIATION IN PHYSIOLOGICAL ACTIVITY

Water Temperature, Growth and Gonad Index

In Fig. 2 are shown the seasonal changes in the

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noontime water temperatures, the shell length, the dry meat weight and the respective gonad index of one or two year old scallops in Onagawa Bay, Miyagi Prefecture (Fig. 3). They were cultivated at a depth of 6 m by the hanging method. The juveniles of these

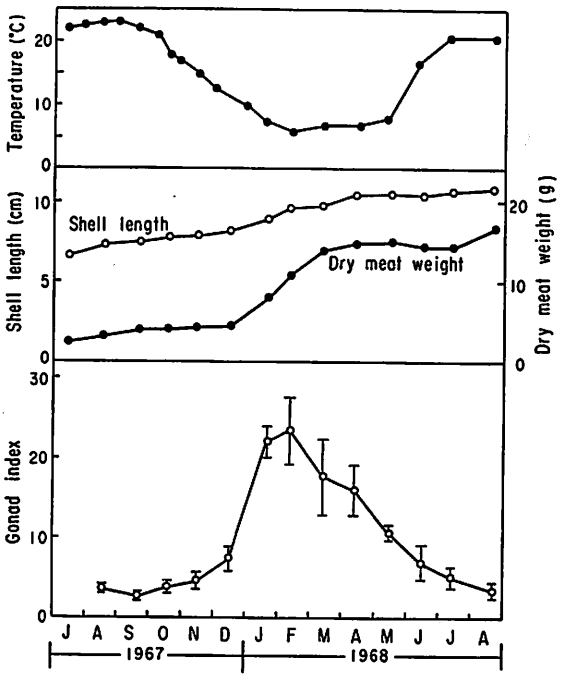

Figure 2. Seasonal changes in the noon-time water temperatures (6 m in depth) observed in the vicinity where scallops were cultivated, the shell length, the dry meat weight and the respective gonad index (dry gonad weight ×100/dry meat weight). The vertical lines are the standard deviations.

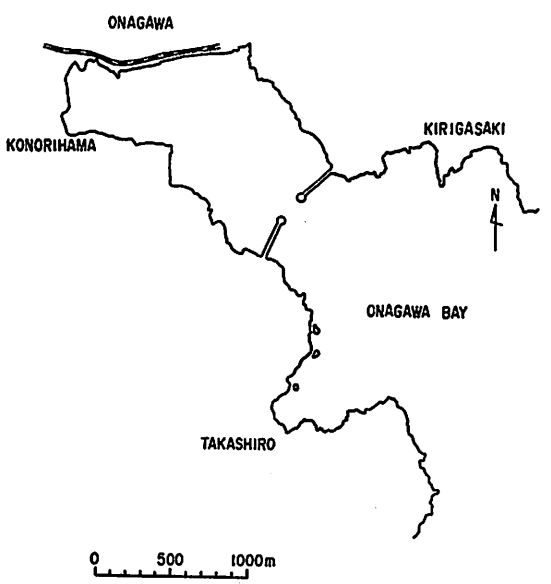

Figure 3. Map of Onagawa Bay.

scallops which had been hatched out of fertilized eggs in May, 1966, at the Mohne Laboratory of the Oyster Research Institute near Kesennuma City, Miyagi Prefecture, were transferred to Onagawa Bay at the end of April in 1967 for this study.

The highest temperature (23°C) was recorded from the middle to the latter part of August in 1967, while the lowest (6°C) was measured in the middle of February in 1968. A sharp increase in soft body weight was noted especially from mid-December to mid-March. The seasonal change in gonad index was negligible before December in 1967, but a sharp increase was found in and after December when the sexes of scallops became distinctly separable with the naked eye. The highest index was seen in the middle of February in 1968 during the period of lowest temperature. After that, the index fell with the rise of water temperature. In short, there was a reciprocal relationship between water temperature and gonad index. It was noticed, in relation to the spawning, that the widest range in the index was obtained in March, 1968, and that a considerable fall in the index was seen from April to May.

Ciliary Activity of Gill

The ciliary activity of gill which has been considered as an indicator for the physiological activity of scallop was measured by the method of Nomura and Tomita (Nomura and Tomita 1933, Tomita 1955). Namely, gill pieces were cut off from the gill lamella and the crawling speed was measured in a graduated straight glass tube. As Fig. 4 shows, this activity was high during the period of low temperature (6–7°C), from February to April, and it was low during high temperature (over 20°C), from July to September. The decline in ciliary activity was far more marked

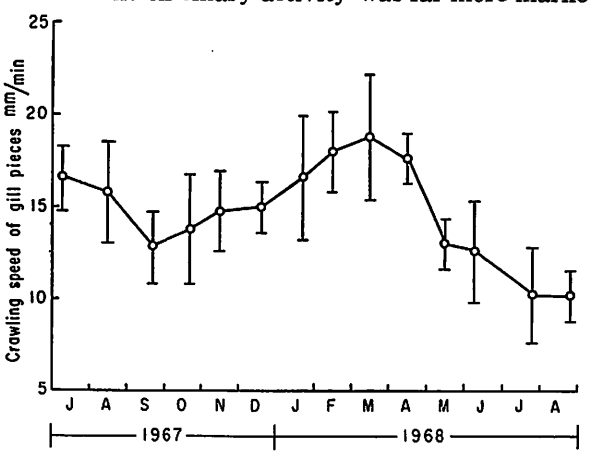

Figure 4. Seasonal change in the ciliary activity of scallop gill. The mean values (circles) and the 95% confidence limits (vertical lines) are shown. Temperature for measurement, 20.0±0.1°C.

in two year old scallops than in one year old ones. This result suggests that the decline in activity during summer may be intensified by the physiological burden related to the reproduction.

Respiratory Metabolism in Tissues

In addition to the crawling speed of gill pieces, the tissue respiration also was investigated to infer the physiological activity of scallop. The experiments were carried out from August to December in 1968. The experimental materials were one year old scallops cultivated in Onagawa Bay whose seeds were artificially collected at the Oyster Research Institute in the spring of 1967 and transferred to this bay late in November. The oxygen consumption in the digestive diverticula, gill, gonad, adductor muscle, pallial margin and kidney was measured using the Warburg apparatus.

The effect of temperature on the oxygen consumption of these tissues from August to September is given in Fig. 5. The order of magnitude of respiratory rate was kidney>gill and digestive diverticula>

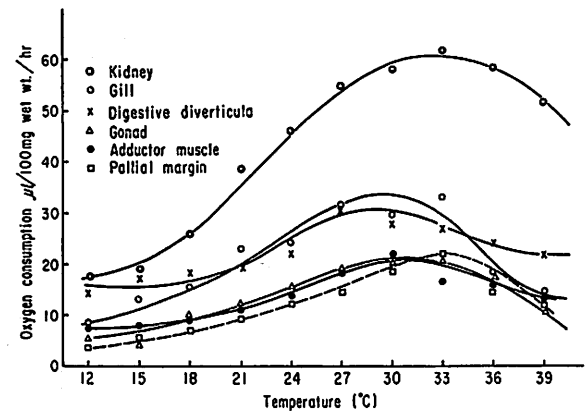

Figure 5. Effect of temperature on the oxygen consumption of various tissues of scallop from August to September.

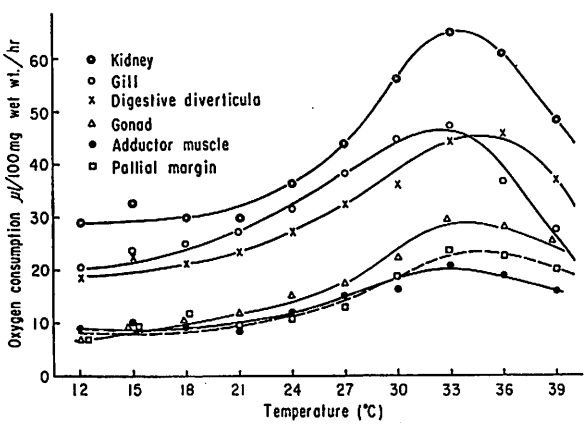

Figure 6. Effect of temperature on the oxygen consumption of various tissues of scallop from November to December.

gonad, adductor muscle and pallial margin. All these tissues formed typical optimum curves. As Fig. 6 indicates, this order was also unchanged from November to December. However, the optimum temperature at this period was not necessarily the same as that from August to September. The difference in the optimum temperature between these two periods was marked especially in the digestive diverticula; 27°C in the former and 36°C in the latter (Fig. 7). This result suggests that the chemical composition of respiratory substrate may change in this organ.

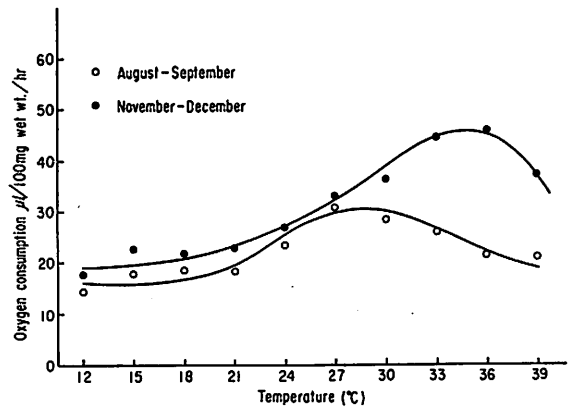

Figure 7. Oxygen consumption of the digestive diverticula of scallop determined from August to September and from November to December in relation to temperature.

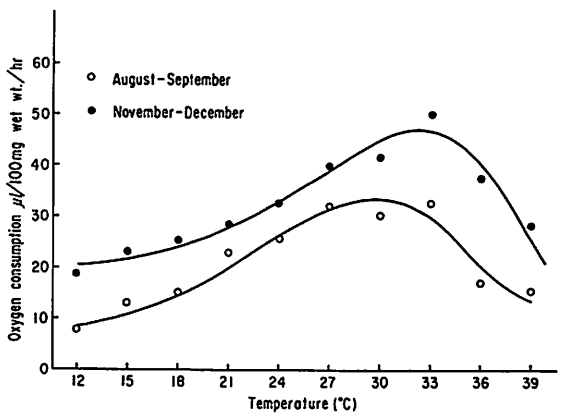

Figure 8. Oxygen consumption of the gill of scallop determined from August to September and November to December in relation to temperature.

In the gill, no significant difference in the optimum temperature was noted between these two periods (Fig. 8). The respiratory rate, however, was higher in November to December than in August to September at all temperatures for measurement, indicating that by December the gill recovers the activity of oxidative metabolism which declined in summer. It is thought that this recovery contributes to the increase in ciliary activity of scallop gill after December.

In Fig. 9 are shown the changes in the oxygen consumption and respiratory quotient (RQ) of the digestive diverticula of one year old scallops from August to December in 1973. They were transferred from Mutsu Bay to Kesennuma Bay (Fig. 10) in December of 1972 and cultivated in one of the non-mortality areas of the latter bay, Hashikami area. The oxygen uptake and carbon dioxide evolution were measured at 20°C using Warburg manometers. Three reaction manometers and one thermobarometer were used in each measurement. The oxygen consumption showed a gradual recovery trend during this experiment from summer to winter. The mean RQ of about 1.3 was observed in August, probably indicating not only the utilization of carbohydrates as the main respiratory substrate but also the active synthesis of fats in this organ. After that, the RQ showed a decreasing trend and dropped down to about 0.7 in December, suggesting the utilization of fats as the main respiratory substrate. In short, the result of Fig. 9 indicates that there is a reciprocal relationship between the oxygen consumption and RQ in the digestive diverticula from August to December. Accordingly, it is supposed that this organ recovers the activity of oxidative metabolism by means of efficient utilization of its own stored lipids as the respiratory substrate.

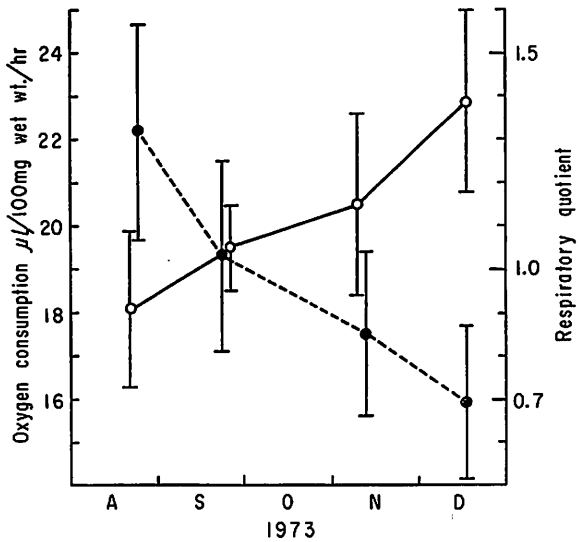

Figure 9. Changes in the oxygen consumption and respiratory quotient of the digestive diverticula of scallop from August to December. The mean values (circles) and the 95% confidence limits (vertical lines) are shown. ○, oxygen consumption. ●, respiratory quotient. Temperature for measurement, 20.0±0.1°C.

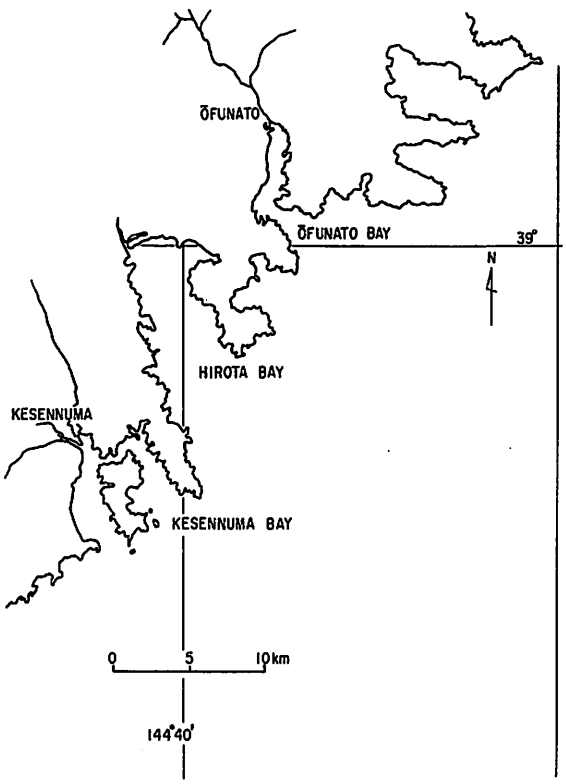

Figure 10. Map of Ōfunato Bay, Hirota Bay and Kesennuma Bay.

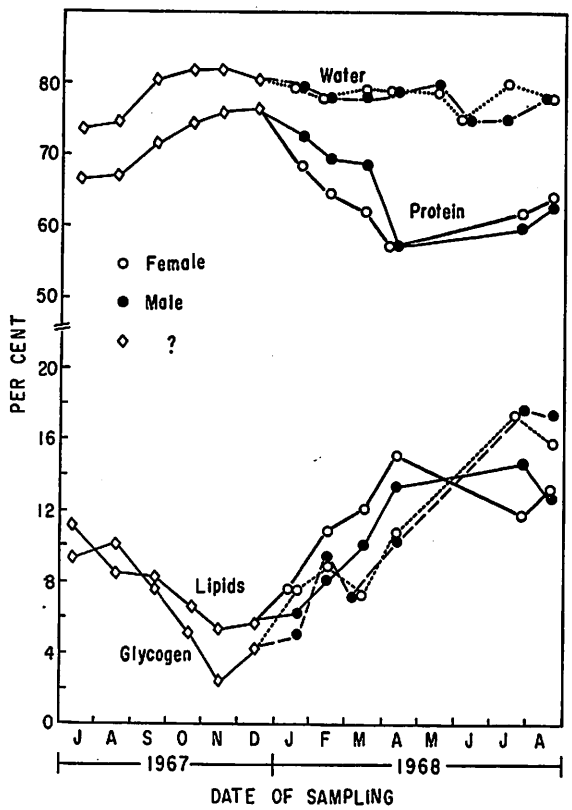

Figure 11. Seasonal change in the chemical composition of the entire soft body of scallop; ordinate, % of soft body wet weight for water, % dry weight for other components. ? ; The sexes were not identifiable with the naked eye.

As given in Fig. 11, the lipid content of the entire soft body of one year old scallop cultivated in Onagawa Bay showed a tendency to decrease from mid-July to mid-November, and was lowest in mid-November when sexual maturation started. This seasonal change was generally parallel to that of the glycogen content. From the chemical analysis of separate body components (Figs. 12 and 13), high concentrations of lipids were found in the digestive diverticula where the glycogen content was very low and exhibited no significant variation through a year. In the middle of August when the water temperature reached the maximum, the lipid content of the digestive diverticula of one year old scallop was 48% of the dry weight (Fig. 12). This percentage corresponds to 79% of the total lipids contained in the entire soft body sampled at the same time. However, it decreased rapidly with the fall in the water temperature and reached 22% late in November when sexual maturation had just commenced, and late in December when the sexes of scallops became separable

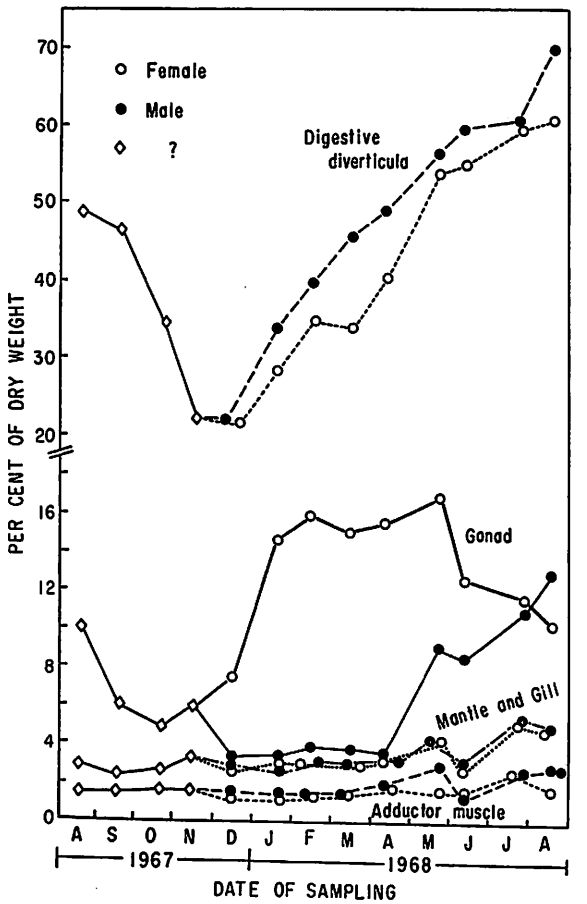

Figure 12. Seasonal variation in the lipid content of separate body components of scallop. ? ; The sexes were not identifiable with the naked eye.

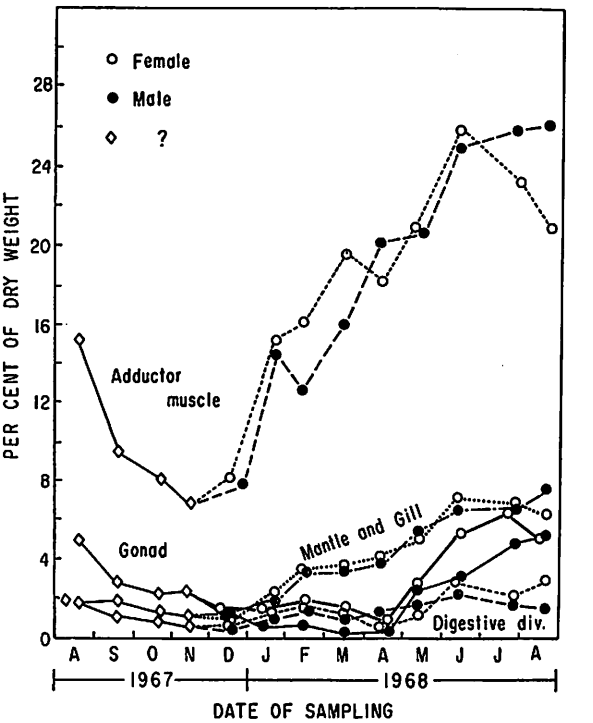

Figure 13. Seasonal change in the glycogen content of separate body components of scallop. ? ; The sexes were not identifiable with the naked eye.

with the naked eye. The percentage (22%) is lower than half of the content determined in mid-August, and it corresponds to 58% of the total lipids contained in the entire soft body sampled at the same time. This rapid decrease in the lipid content from August to December supports the previous idea that lipids are increasing in importance as the respiratory substrate in the digestive diverticula during this period (Fig. 9). Hence, it is thought that scallops depend on the lipids accumulated in this organ in summer for main energy source in order to recover their physiological activity for the coming rapid development of their gonad after December.

LIMITS OF THE TOLERANCE TO HIGH TEMPERATURES

In Tables 1 and 2 are shown the Q_{10} values calculated at intervals of 3°C on the oxygen consumption of various tissues of one year old scallops from August to September and from November to December. These experimental materials are just the same that were used in Figs. 5 and 6. It was found that the "Q_{10}-Regel" of van't Hoff applied fairly well to the lower range than the optimum temperature.

The relation of the difference between the maximum and minimum of Q_{10} values to the temperature range is summarized in Fig. 14. At either period,

this difference showed its minimum in the temperature range of 21–24°C. This difference, namely, the measure of dispersion of Q_{10} values represents approximately the degree of discrepancy in irritability to temperature among the tissues examined. Therefore, it is thought that these tissues do not function organically under the condition of higher temperatures than 21–24°C and then the physiological catastrophe occurs in the scallop.

In the customary method for determining the limits of the physiological tolerance of aquatic animals to high temperatures, intact animals are kept under the temperatures for test. Accordingly, the limits obtained often vary with the temperature conditions where experimental materials lived before test. However, the tolerance limits of scallop at the two periods revealed by means of the present method coincide with each other, in spite of the considerable difference in the effect of temperature on the tissue respiration between these periods (Figs. 5 and 6). This suggests that the effects of temporary irritability or acclimatization which disturb the determination of the true limits of tolerance are eliminated in the present method.

Table 1. Q_{10} values calculated at intervals of 3°C on the oxygen consumption of various tissues of scallop from August to September.

Temperature (°C)	Kidney	Gill	D. d.	Gonad	A. m.	P. m.
12–15	1. 31	2. 36	1. 00	2. 23	1. 24	3. 28
15–18	2. 29	2. 81	1. 22	2. 31	1. 48	3. 07
18–21	3. 07	2. 61	1. 58	2. 61	1. 95	2. 77
21–24	2. 31	2. 74	2. 43	2. 53	2. 51	2. 18
24–27	1. 84	1. 66	1. 72	2. 15	2. 25	2. 35
27–30	1. 38	1. 23	1. 06	1. 28	1. 41	1. 97
30–33	1. 09	0. 34	0. 65	0. 92	0. 92	1. 76
33–36	0. 89	0. 24	0. 55	0. 53	0. 52	0. 56
36–39	0. 66	0. 30	0. 86	0. 20	0. 51	0. 18

D. d.=Digestive diverticula, A.m.=Adductor muscle, P.m.=Pallial margin

Table 2. Q_{10} values calculated at intervals of 3°C on the oxygen consumption of various tissues of scallop from November to December.

Temperature (°C)	Kidney	Gill	D. d.	Gonad	A. m.	P. m.
12–15	1. 07	1. 08	1. 19	1. 56	0. 89	0. 82
15–18	1. 06	1. 56	1. 18	2. 10	1. 21	1. 22
18–21	1. 23	1. 57	1. 46	1. 84	1. 42	1. 31
21–24	1. 55	1. 66	1. 59	1. 67	1. 84	1. 63
24–27	1. 86	1. 93	1. 86	1. 91	1. 88	2. 23
27–30	2. 23	1. 50	1. 76	2. 74	2. 06	2. 53
30–33	1. 69	1. 25	1. 68	2. 17	1. 41	2. 22
33–36	0. 97	0. 58	1. 04	0. 87	0. 85	0. 93
36–39	0. 48	0. 25	0. 53	0. 64	0. 62	0. 68

Abbreviations are the same as in Table 1.

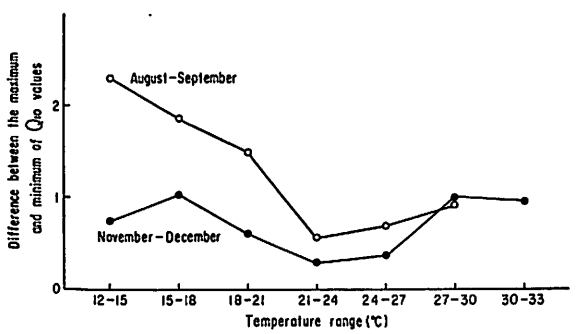

Figure 14. Relation of the difference between the maximum and minimum of Q_{10} values calculated on the oxygen consumption of various tissues of scallop to the temperature range.

MASS MORTALITY

General View

In 1972, no data are available on the exact mortality rate of juvenile scallops which were transferred from Mutsu Bay to Iwate Prefecture in November or December of 1971 and cultivated there. However, it is speculated that over 50% of the approximately 60

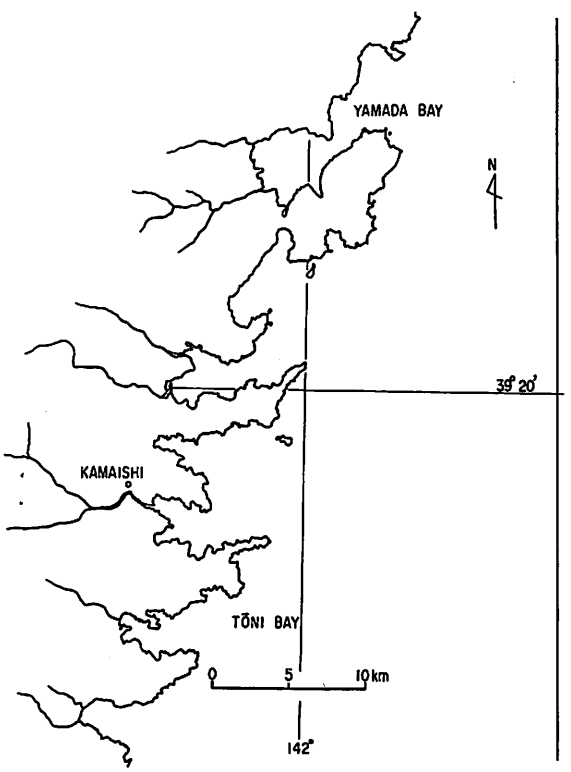

Figure 15. Map of Yamada Bay and Tōni Bay.

million scallops transferred to Yamada Bay (Fig. 15) died in 1972. Such large-scale deaths of scallops are also speculated to have occur in Ōfunato Bay and Hirota Bay (Fig. 10) in the same year. The scallop production from the two bays of Yamada and Ōfunato accounts for 90% (in the fiscal 1971) and 83% (in 1972) of all the crop in Iwate Prefecture. Hence, the mass mortality of scallop in this prefecture became an object of public concern.

In 1973, the detailed investigation on the mortality rate of the scallops under culture there was conducted by the authorities concerned of the Iwate prefectural government. According to it, the mortality rate up to December of 1973 of juvenile scallops which were transferred from Mutsu Bay in 1972 and cultivated was 67% in Yamada Bay, 85% in Ōfunato Bay, 80% in Hirota Bay and 90% in Miyako Bay (Iwate Prefectural Fisheries Promotion Section 1974).

In Kesennuma Bay of Miyagi Prefecture, one year old scallops began to die from the end of May in 1972 and then two year old ones also died in hot summer. It is estimated that this bay lost 87% of juvenile and adult scallps, namely, 17 million individuals up to the end of August in 1972 (Miyagi Prefectural Kesennuma Fisheries Experimental Station 1972). In 1973, the mass mortality occurred not only in Kesennuma Bay but also in Shizugawa Bay near Kesennuma.

Moribund scallops in these farms were commonly characterized by the deformity or incision of shell margin, the adhesion of brown film-like substance on the inside of shell margin, and the atrophy of pallial margin (Miyagi Pref. Kesennuma Fish. Exp. Sta. 1972, Egusa 1973, Iwate Pref. Fish. Pro. Sec. 1973). At the period of mass mortality, however, it was difficult to find out some common environmental factors which were thought to be directly connected with its occurrence. Hence, it is suggested that the analytical studies on various factors related to the decline in physiological activity of young scallop during the period from late spring to early summer when the mass mortality occurs are needed together with more detailed environmental investigations in order to clarify its causative agents.

Hypothesis on a Possible Cause

From the above-mentioned general view of the mass mortality, our preliminary biological observations on moribund scallops found in various farms and reproduced artificially in some experimental conditions, and some environmental data, the author prepared a schematic diagram illustrating a hypothesis on the physiological progress until death of juvenile scallop under hanging culture in the coastal waters of Sanriku district (Fig. 16). This hypothesis was advanced at the Meeting for Intermediary Report of Studies on Scallop Mortality held in Kamaishi in

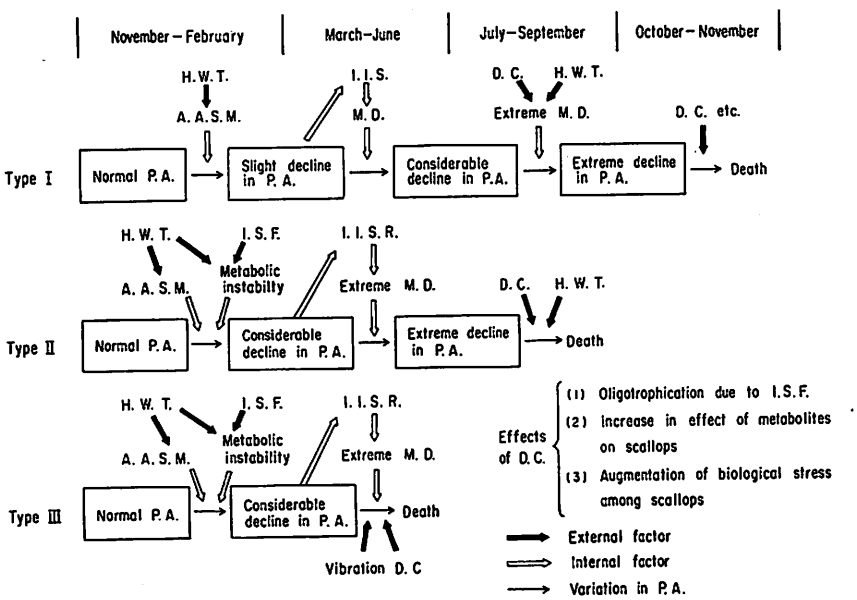

Figure 16. Schematic diagram illustrating a hypothesis on the physiological progress until death of juvenile scallop under hanging culture in the coastal waters of the Sanriku district. P. A., physiological activity. H. W. T., higher water temperatures than usual. M. D., metabolic disturbances. A. A. S. M., abnormal acceleration of sexual maturation. I. I. S. R., incomplete or impracticable spawning and reabsorption. I. I. S., incomplete or impracticable spawning. D. C., dense cultivation. I. S. F., insufficient supply of foods.

November of 1973. As has been stated, the physiological activity of scallop changes with the season or its growth even under normal environmental conditions. Accordingly, the term "normal activity" appeared in this diagram means the level of activity that a healthy scallop of the same age is expected to show at the same season under normal environment. The term "decline in activity" means that the activity declined to a lower level than the above-mentioned one.

This hypothesis consists of three types of progresses (I–III) that differ in the period of death. What is common to these progresses is the supposition that abnormal acceleration of sexual maturation of juvenile scallops and the subsequent disorder of sexual cycle resulting from incomplete or impracticable spawning due to insufficient maturation of their gonads and other factors may bring about various types of metabolic disturbances under the influence of dense cultivation and others. It can readily be imagined that the abnormal acceleration of sexual maturation in the coastal waters of Sanriku district is caused by such environmental conditions as the higher water temperatures in winter than those observed in Mutsu Bay and northward.

An increase in effect of metabolites on scallops and an augmentation of biological stress among them as well as the oligotrophication due to insufficient supply of foods are supposed as the effects of dense cultivation in this hypothesis.

Substantiation of This Hypothesis

(1) Effect of Higher Water Temperatures than Usual on the Sexual Maturation of Juvenile Scallop in Winter

In the coastal waters of Sanriku district, the temperatures observed both from the winter of 1971 to the spring of 1972 and from the winter of 1972 to the spring of 1973 were higher than usual (Iwate Pref. Fish. Exp. Sta., unpublished data) and the mass mortality occurred from spring to early fall in both 1972 and 1973 mainly among one year old scallops under culture. However, this region experienced extremely low water temperatures which were caused by unusually close proximity of a cold water mass, from mid-February to late April in 1974, after this hypothesis had been advanced (Fig. 17). It is thought that such low temperatures can suppress the sexual maturation of juvenile scallops. Hence, it is expected that there can be a small mortality in 1974, if this hypothesis is reasonable. Really this expectation has come true, though other environmental conditions such as high temperatures in hot summer this year are similar to those in 1973. This indicates that

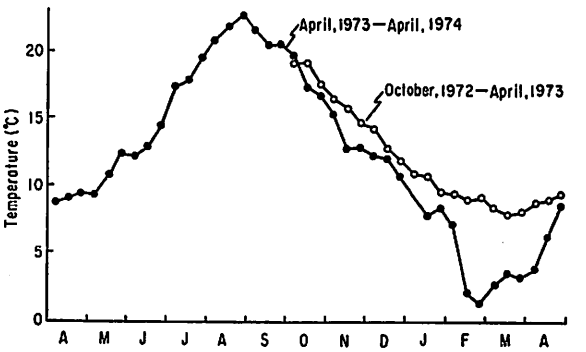

Figure 17. Seasonal changes in the mean water temperatures (0 m in depth) in Tōni Bay.

higher water temperatures in winter constitute a very important factor in the occurrence of mass mortality of juvenile scallops.

In 1973, Mori and Osanai (1974a, b) carried out histological studies on the seasonal variation in gonad of scallops under culture in Tōni Bay and Yamada Bay (Fig. 15) and showed that gonadal maturation proceeded rapidly during winter even in scallop seeds under one year of age, though their sex differentiation was unstable. In Mutsu Bay of Aomori Pref. and Funka Bay of Hokkaido, sexual maturation in such scallop seeds was observed to be suppressed in winter (Mori et al. 1974a).

During the period from November to January, the sexual maturation of juvenile scallop proceeds more rapidly in Yamada Bay than in Tōni Bay (Mori and Osanai 1974a, b, Mori et al., unpublished data). This may be successfully explained in terms of the difference in condition of food supply (Hirose 1972, Tsujita 1973, Mori et al. 1973, Mori et al., unpublished data).

(2) Insufficient Supply of Foods and Metabolic Instability

Usually, the condition of food supply in Tōni Bay and Yamada Bay reverses itself during the period from February to March. In Tōni Bay, this condition takes a turn for the better (Mori et al., unpublished data). In Yamada Bay, however, the standing crop of planktons becomes extremely small (Hirose, 1972) and an impediment ring and incision are formed on the shell of juvenile scallop during this period. Physiologically, it seems probable that such an insufficient supply of foods from February to March makes the metabolism of juvenile scallops unstable, because their need for feeding has been intensified by both the abnormal acceleration of sexual maturation from November to January and higher water temperatures in winter than usual. Consequently the scallops exhibit a considerable decline in physiological activity.

On the other hand, it is likely that this insufficient supply of foods to juvenile scallops is partially due to the active intake of foods by a tremendous crowd of adult scallops under culture whose gonads are rapidly developing just from February to March (Mori et al. and Iwate Pref. Fish. Exp. Sta., unpublished data). This is one of the most noteworthy problems for the culture management of scallop in the coastal waters of Sanriku district.

(3) Incomplete or Impracticable Spawning and Reabsorption and Metabolic Disturbances

In general, spawning is observed from April to May in the coastal waters of Sanriku district. During this period of 1973, however, little or no spawning was found in juvenile or young scallops under culture in Yamada Bay where the mass mortality occurred in summer. Besides, there were a considerable number of individuals possessing many mature eggs or sperms even during the immature stage from July to August. This fact indicates that the reabsorption of their gonads was incomplete or impracticable (Mori and Osanai 1974b). In Tōni Bay, on the other hand, the reabsorption proceeded normally, though there were some juvenile scallops exhibiting incomplete spawning (Mori and Osanai 1974a).

The occurrence of various types of metabolic disturbances in juvenile or young scallops of Yamada Bay was verified or suggested by our experiments. The mantle showed a disturbance of amino acid metabolism (Mori and Kashiwagi 1974). A disorder of lipid metabolism was suggested in the digestive diverticula (Ito, Hata and Mori 1974a, b). A disturbance of glycogen metabolism was suggested in the adductor muscle (Mori et al. unpublished data). Table 3 summarizes some data from our analysis of free amino acids in the mantle tissue. It is worth noting that their content in Yamada Bay was extremely low in August when very high mortality was experienced.

Table 3. Free amino acids (μmoles/g wet weight) in the mantles of one-year-old scallops under hanging culture in the coastal waters of Sanriku district.

Amino acid	June		August	
	T	Y	T	Y
Lysine	0.231	trace	0.282	trace
Histidine	0.109		0.152	trace
Arginine	4.562	4.248	4.098	1.415
Aspartic acid	0.236	0.330		0.100
Threonine+Glutamine	0.902			
Serine+Asparagine	0.469	0.256	0.304	0.198
Glutamic acid	2.546	1.838	1.742	0.558
Proline	0.404			
Glycine	129.795	104.052	113.238	39.154
Alanine	6.054	4.312	4.300	0.217
Valine	0.166	trace	0.306	trace
Methionine	0.408		0.376	
Isoleucine	0.105			
Leucine	0.198			
Phenylalanine	0.467			

T=Tōni Bay, Y=Yamada Bay

These metabolic disturbances were frequently found particularly in some farms where scallops were densely cultivated, revealing that they occur under the influence of dense cultivation. In such farms, water pollution was often observed more or less especially from May to September (Mori and Fujita 1974). This seems to be mainly due to the metabolites from numerous scallops cultivated there.

(4) Experimental Reproduction of Abnormal Mortality

If this hypothesis is reasonable, it is expected that the abnormal mortality is reproducible from May to July under such experimental conditions of oligotrophy or oligotrophy plus a certain stress accelerator. The author and his coworkers (Mori et al. 1974b) tried such an experiment using a large-sized indoor tank in the Miyagi Prefectural Kesennuma Fisheries Experimental Station and obtained the result shown in Table 4. One year old scallops transferred from Mutsu Bay to Kesennuma Bay in December of 1972 were used as the materials. The control animals were kept in one of the non-mortality areas of the latter bay, Hashikami area. In this experiment, oligotrophy was regarded as one of the effects of dense cultivation. Transversal vibration (amplitude, 20cm; frequency, 25 times/min.) was given to scallops for 2 minutes every day, because this was supposed as one of accelerators to augment the biological stress caused by the condition of hanging cultivation. Besides, the augmentation of biological stress among scallops was imagined to be more strongly accelerated under the dense cultivation, so the experimental condition of oligotrophy plus vibration was employed.

As Table 4 shows, the growth in the experimental groups was decidedly inferior to that in the control. Scallops in the experimental groups showed high mortality of 48% or 60%, while the control animals had very low mortality of less than 3%. Moribund scallops exhibited the deformity or incision of shell margin, the adhesion of brown film-like substance on the inside of shell margin, and the atrophy of pallial margin (Figs. 18 and 19). These outward symptoms are exactly the same as those given by moribund scallops in several farms of the Sanriku district.

Table 4. Growth and mortality of scallop under two kinds of experimental conditions, oligotrophy and oligotrophy plus vibration. Experimental period: May 1 to July 25, 1973

Items			Experimental conditions		
			Control	Oligotrophy	Oligotrophy plus vibration
Shell length	mm	Average at the end of experiment	73 (25.9)[1]	64 (10.3)	58 (0)
Shell height	mm		68 (25.9)	58 (7.4)	55 (1.9)
Shell width	mm		20 (33.3)	17 (13.3)	16 (6.7)
Shell weight	g		26.5(107.0)	16.3(27.3)	14.9(16.4)
Wet meat weight	g		17.7(80.6)	8.0(−18.4)	7.0(−28.6)
Final mortality rate	%		2.6	48.3	59.8

[1] Parenthesized numbers show the rate of increase (%) during the experiment.

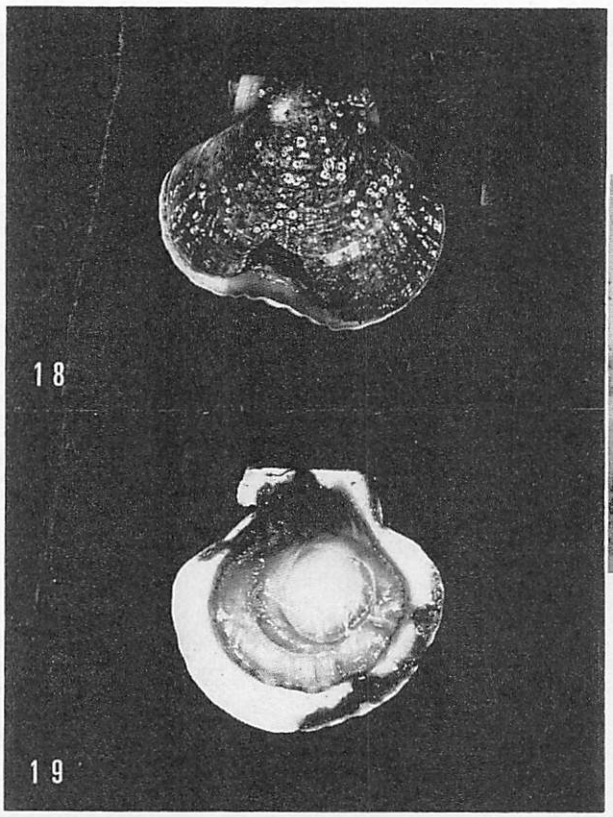

Figure 18. An experimental scallop showing the incision of shell margin. The opposite shell margin exhibits an anomalous growth. ×0.6

Figure 19. An experimental scallop showing the adhesion of brown film-like substance on the inside of shell margin. The atrophy of pallial margin is so conspicuous that the eyes are hardly able to be distinctly observed. ×0.6

Fig. 20 is the picture showing a general view of the mantle of an abnormal scallop. Serious pathological changes were also observed in the digestive diverticula (Figs. 21 and 22) and adductor muscle (Figs. 23 and 24) of the same scallop. No pictures suggesting a bacterial infection or parasitic invasion were found in the organs examined. These histological observations are in accord with those of abnormal scallops sampled in the farms where the mass mortality occurred.

From the above-mentioned facts, experimental results and discussion, it may be concluded that our

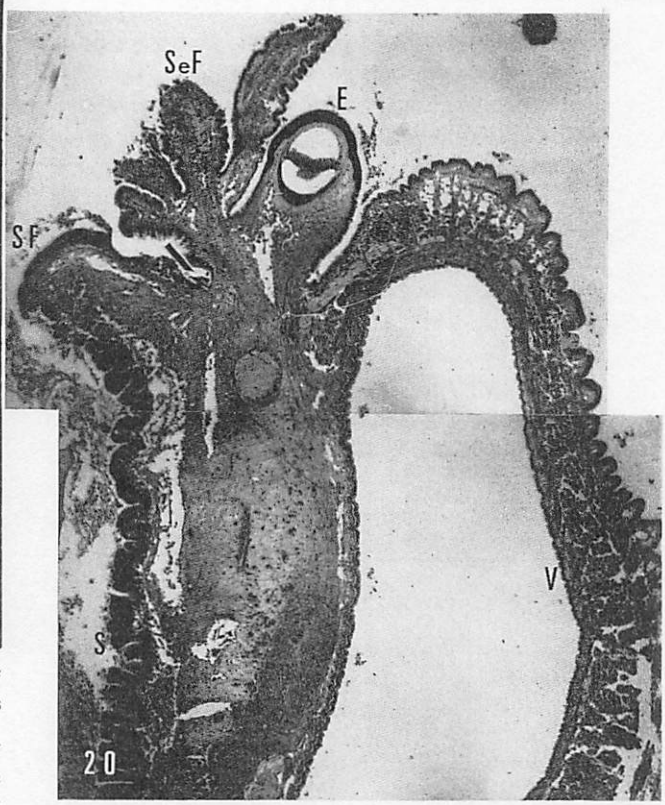

Figure 20. General view of the abnormal mantle of an experimental scallop. Inflammation, separation and falling-off are observed in the epithelia of the sensory fold (SeF) and shell fold (SF). Remarkable folding is found in the shell-side epithelia (S) of the mantle. This folding and the roundness at the tip of shell fold are caused by the atrophy of pallial margin. The marked infiltration of amoebocytes is seen in the connective tissues of the above-mentioned two folds (SeF, SF) and in the mucous membranes of the shell-side epithelia (S). No secretion from the periostracal gland (arrow) is recognized. E—eye. V—velum. Hematoxylin-eosin (H-E) stain. ×36.

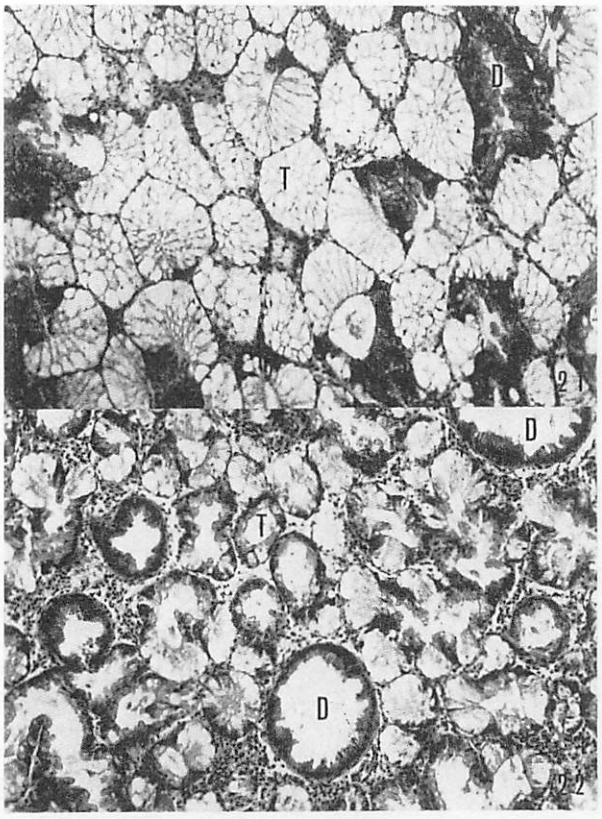

Figure 21. Digestive diverticula of a control scallop sampled on July 25. Most of the tubules consist of a large number of highly developed fat cells. D—duct. T—tubule. H-E stain. ×90

Figure 22. Digestive diverticula of an experimental scallop sampled on July 25. Few tubules (T) possess many well-developed fat cells. The lumina of the ducts (D) are enlarged. An intense amoebocytic infiltration is observed in the connective tissues. H-E stain. ×90.

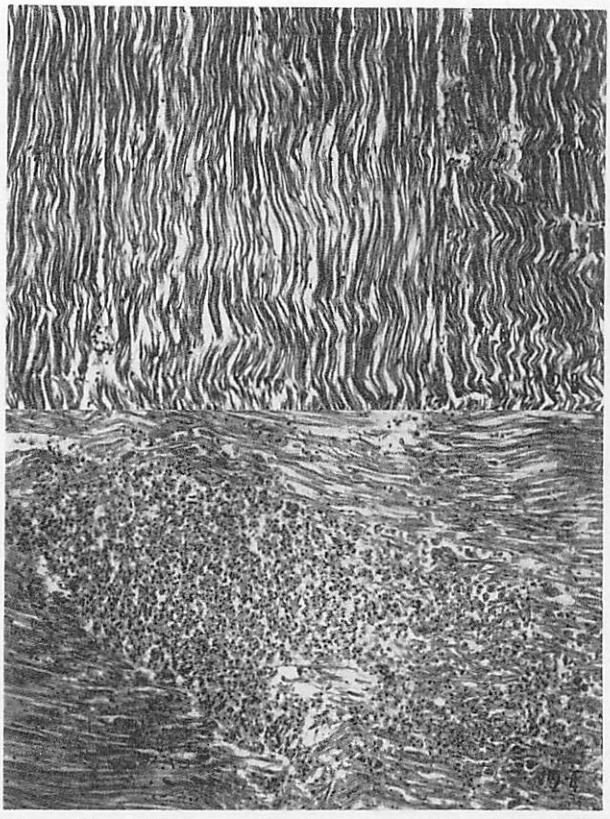

Figure 23. Adductor muscle of a control scallop. No pathological change is found. H-E stain. ×90.

Figure 24. Adductor muscle of an experimental scallop. The inflammation of muscle fibers and the hyaline degeneration are clearly recognized. H-E stain. ×90.

hypothesis on the possible cause of the mass mortality of juvenile scallops under hanging culture in the coastal waters of the Sanriku district is reasonable.

Countermeasures for Mass Mortality

According to our hypothesis (Fig. 16), the following subjects can be proposed as the tentative countermeasures for the mass mortality of juvenile scallops.

(1) Reexamination of the time when scallop seeds are transferred to the coastal waters of the Sanriku district.

(2) Prevention of excessively dense cultivation of scallops and thorough conservation of scallop-farm's environment.

(3) Development of the equipment that seldom gives a vibration to scallops under hanging culture.

Concerning the first subject, an introduction of semi-adult seeds instead of juveniles, for example, may be acceptable as a countermeasure. The author has already proposed to make a few bases for semi-adult seed supply in the coastal waters of the Sanriku district (Mori, 1974). Tōni Bay is expected to be one of the bays available as such bases.

Regarding the second subject, it is most important for us to find as soon as possible how to estimate justly the number of scallops that can be normally cultivated in each bay, though this is very difficult because each bay has its own environmental characteristics and the environmental conditions are changeable even in the same bay. The Iwate Prefectural Fisheries Experimental Station is trying to estimate this number of scallops in each bay of Iwate Prefecture on the base of the P/S ratio of Mori and Hirose (1974) in order to guide fishermen in their prevention of excessively dense cultivation of scallops.

Concerning the third subject, at least, the conversion of the present raft culture to the long-line culture is needed in Yamada Bay, Ōfunato Bay and Kesennuma Bay where the mass mortality occurred, because the long-line method possesses the advantage of withstanding winds, waves, and currents better than the raft method.

SUMMARY

The present study was carried out in order to investigate the seasonal change in physiological activity of scallop, *Patinopecten yessoensis*, under culture in the coastal waters of Sanriku district, Japan and to clarify the causative agents of their mass mortality.

The physiological activity revealed by the crawling speed of gill pieces was high during the period of low water temperature (6–7°C), from February to April, and it was low during high temperature (over 20°C), from July to September. The decline in activity during summer was suggested to be intensified by the physiological burden related to the reproduction. This seasonal change in physiological activity was confirmed by the manometric investigation. In addition, the metabolic significance of respiratory substrate in tissues was discussed in relation to this seasonal change.

From the manometric investigation it was concluded that the tissues of scallop do not function organically under the condition of higher temperatures than 21–24°C and then the physiological catastrophe occurs in the scallop.

From the general view of the mass mortality, our preliminary biological observations on moribund scallops and some environmental data, the author prepared a schematic diagram illustrating a hypothesis on the physiological progress until death of juvenile scallop under hanging culture in the coastal waters of Sanriku district. This hypothesis consists of three types of progresses that differ in period of death. What is common to these progresses is the supposition that the abnormal acceleration of sexual maturation of juvenile scallops and the subsequent disorder of sexual cycle resulting from incomplete or impracticable spawning due to insufficient maturation of their gonads and other factors may bring about various types of metabolic disturbances under the influence of dense cultivation and others.

The substantiation of this hypothesis was tried and it was found that this hypothesis is reasonable. Finally, three tentative countermeasures for the mass mortality of juvenile scallops were proposed according to this hypothesis.

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NOCARDIAL INFECTION IN CULTURED YELLOWTAILS

RIICHI KUSUDA[1]

INTRODUCTION

In Japan the cultivation of marine fishes made a rapid progress in the last several years, and now comes to hold an important part of fisheries. However, there are many unresolved problems in the cultivation of fishes. Especially, the loss of rearing fishes due to infectious disease is one of the largest obstacles to the further development of this industry. In spite of the general inference that a large portion of the disease occurring prevalently on rearing fishes is probably attributable to the infection of pathogenic bacteria to the fishes, little work has been done on the bacterial disease of marine fishes.

The present paper outlines our investigation on nocardial infection of cultured yellowtails, *Seriola quinqueradiata* and *S. purpurascens*, caused by *Nocardia kampachi*, with special references to the characteristics and pathology of the disease, the haematology, the characteristics of the causative organism, and the prevention method of the disease.

CHARACTERISTICS AND PATHOLOGY OF THE DISEASE

The disease of yellowtail now known as nocardial infection was first recorded by Kariya *et al.* (1968), who stated that during the late summer and autumn of 1967, many yellowtails from the Mie Prefecture were found to have small abscess like lesions on their skin. From these lesions a branching gram positive bacillus could be isolated. Since then the severe prevalence of the nocardial infection occurred often at the rearing farms of marine fishes in various districts of Japan.

The disease attacks the cultured yellowtails, *Seriola quinqueradiata* and *S. purpurascens*. It is characterized by slowness of the course and severity of mortality rate.

The clinical signs shown by yellowtails in the early stages of nocardial infection may include emaciation, inactivity and discoloration of the skin. There are followed by tubercle of the body surface and formation of abscess. They are rounded, approximately 5 mm in diameter, slightly raised. The abscess later become tubercular ulcerative. There seem to be no diagnostic external symptoms of the disease in the older age group, but fish suffering from this disease may show large creamish-white tubercles on the gill surface, haemorrhage at the lips and, in a very few cases, haemorrhagic spots on the body surface. The terms "gill tuberculosis" (Kusuda *et al.*, 1974), "palatine tuberculosis" and "red spot tuberculosis" have previously been employed to describe nocardia-associated diseases in yellowtails. Such symptoms which are not found in other fish diseases are of much value in the diagnosis of nocardial infection.

Internally the yellowtail has very characteristic lesions, the most prevalent one being abscess of the muscular and sub-cutaneous tissues, where they have caused some degree of caseation. Those abscess are very common. The next organ most often affected is the spleen. The kidney and swim bladder are the next in order. A number of nocardial nodules are seen in the spleen, swim bladder and kidney, and less frequently in the liver and pericardium. The nodules are creamish-white, measured in size from pin-head to about 4 mm in diameter, and are firm to the touch, and contrasted markedly with the appearance of normal healthy tissue in these organs. The appearance of the stomach and intestine is normal.

Microscopical examination was extended to include histological preparations of the spleen and kidney. In both the spleen and the kidney nodules can be seen. The lesion are of a tubercular nature, and many of them gave the appearence of healing. The most characteristic structures found are bacillary masses lying within small cavities bounded by concentrically arranged fibrous tissue. This fibre formation is also observed in the smallest nodular masses, and is thought to represent an encapsulation of the tubercular areas. The capsules are composed of fibrous tissue arranged concentrically around the

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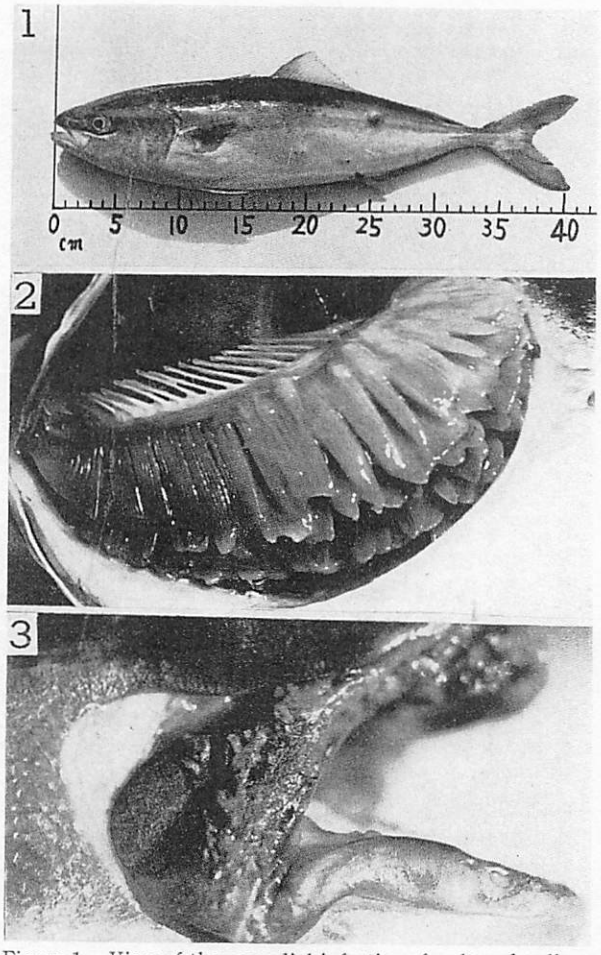

Figure 1. View of the nocardial infection of cultured yellowtails showing typical lesions caused by *Nocardia kampachi*. 1: abscess-tuberculosis type, 2: gill-tuberculosis type, 3: palatine-tuberculosis type.

bacteria with small and large connective tissue nuclei and relatively coarse fibres. These circular areas of focal necrosis are sharply circumscribed, and apparently bore little relation to the vascular supply. The spleen and the kidney cells are occasionally replaced by large mononuclear cells in many of which the nuclei have disappeared. At the periphery of the areas of necrosis occasional giant cell are detected. These have large nuclei, and the adjacent spleen and kidney cells are granular, swollen, and possessed ill-defined nuclei. The outline of the spleen and kidney cells is irregular, and the blood vessels are distended. Numerous clumps of feebly acid-fast bacteria are found in the necrotic areas, and smaller groups of organisms occurred within intra-vascular masses. There are a number of large mononuclear cells which contain varying numbers of feebly acid-fast bacteria often replacing the cytoplasm itself.

HAEMATOLOGY

On the blood changes observed in yellowtail affected by nocardial infection from the western of Japan are given in Table 1.

Table 1. Haematological changes observed in yellowtail affected by nocardial infection.

Diseased type	Haemoglobin (g/dl)	Erythrocytes ($\times 10^4$/mm^3)	Haematocrit (%)
Abscess tuberculosis			
1. Normal (0 age)	12. 7	291	6. 15
2. Diseased (0 age)	8. 2	202	5. 82
Gill tuberculosis			
1. Normal (1 age)	13. 9	382	6. 38
2. Diseased (1 age)	7. 0	207	4. 24

From the observation, it is apparent that fish suffering from nocardial infection show a lowering of the haemoglobin concentration, erythrocyte count and haematocrit consistent with a marked anaemia.

ISOLATION OF THE CAUSATIVE ORGANISM AND ITS PATHOLOGENITY TO FISHES

From the lesions of muscle and internal organs of diseased fishes, many strains of bacteria were isolated by the use of Ogawa's medium (egg-media). Almost all of the cultures obtained were exactly identical one another in morphological, cultural and physiological characteristics.

The representative culture of isolated bacteria tested for the pathogenity to fishes. A suspension was prepared by adding about 6mg of the Ogawa's agar slope culture in 10ml of sterilized physiological salt solution and its small quantity was inoculated to healthy yellowtail (9–11cm in body length) by menas of intramuscular injection, oral inoculation, a smearing the skin or an addition to the environmental water. A large part of the fishes tested was slowly infected with the disease and fell dead within 4 to 20 days. They exhibited the same symptoms as yellowtail naturally suffering from the nocardial infection in the external appearance and the internal organs. The pure cultures of the same bacterium as inoculated were obtained without exception from the spleen and kidney of the fishes that were experimentally affected.

The organism was also pathogenic for carp, *Cyprinus carpio*.

It was also observed in the infection experiment that susceptibility to the disease of fish was remarkably affected by the method of inoculation of the bacterium. The injection of the bacterium into

musculature exhibited the highest pathogenicity. Smearing incised wound on the body surface with the bacterium also caused severe attack. The oral inoculation method was the next in order. Smearing the normal skin with the bacterium or adding it into a rearing water gave no infection. From these results, it is considered that the infection through the alimentary canal as well as through the wounded parts on the body surface is practically serious.

CHARACTERISTICS OF THE ORGANISM

The main characteristics of the causative organism of the disease are as follows.

Morphology

Non-motile gram-positive rods of various lengths and short filaments which occasionally showed slender branching filaments, and were feebly acid-fast. They were non-sporning, and produced aerial hyphae.

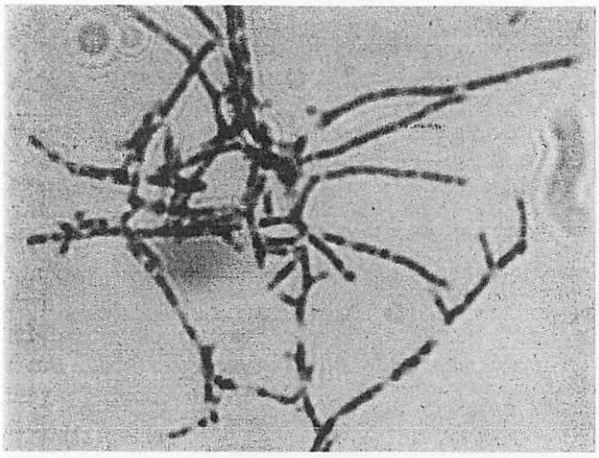

Figure 2. Photomicrograph of *Nocardia kampachi* strain N-1, cultured in Dubos's broth. Show branching. ×1,500.

Growth on Agar Plate

The growth of the cultures on slant of Ogawa's agar, after 10 days of incubation at 25°C, varied from thin and restricted to abundant and spreading. It was flat, finely or coarsely wrinkled, or warty.

Biochemical Characters

Catalase, hydrogensulfide production, starch hydrolysis, and nitrate reduction were positive. Decomposition of hypoxanthine and tyrosine was feebly positive. Tests for oxidase, urease, indole production, gelatin liquefaction, and decomposition of casein and xanthine were negative. They were able to produce acids from glucose, fructose, and glycerol on a medium containing sodium glutamate as the nitrogen source. The organisms utilized the acetate, citrate, fumarate, and pyruvate, but not the benzoate, oxalate, propionate, and tartrate on a medium containing diammonium hydrogen phosphate as the nitrogen source.

Biological Characters

Aerobic. Optimum temperature for growth appeared to be 25–28°C, and growth ocured at 12–32°C. No growthed at 10°C and 37°C. Optimum pH for growth appeared to be 6.5–7.0, and growth was possible at the pH range 5.8–8.5. Optimum salt concentration for growth to be 0–1.0%, and growth was possible at the concentration range 0–4.0%. The organisms showed growth neither at 10°C nor 37°C and survived at 50°C for 4 hours in the nutrient broth.

The organisms had much in common with those of *Nocardia kampachi*, but could be distinguishable from those of *Nocardia asteroides*.

IMMUNOLOGY

The development of agglutination antibody and protective immunity to the organism in yellowtail was examined by serological techniques. It was accomplished by double intramuscular injection of heat-killed and formalin-killed bacteria or formalin-killed adjuvant vaccine. The experiment results were given in Table 2.

Table 2. Immune response of yellowtail to *Nocardia kampachi*.

Antigens	Mean titer after injection (weeks)				
	1	3	5	8	12
Heat-killed	1, 152	7, 168	10, 240	4, 352	1, 024
Formalin-killed	2, 816	7, 168	11, 264	6, 144	768
Formalin-killed +Adjuvant	2, 688	6, 144	9, 216	5, 120	1, 024
Control	10	15	15	15	50

The response of yellowtail to nocardial antigenic stimulation was slowly. Maximum agglutinin titers occurred 5 weeks after injection.

PROPHYLAXIS AND THERAPY

Antibiotics and bacteriostatics have been used to prevent and control outbreaks of bacterial infection in marine fish, but no information available with specific reference to nocardiosis. Then, the antibiotic sensitivity *in vitro* of a strain of *Nocardia* isolated from the disease in yellowtail was examined by methods of serial dilution or resistance test. Some examples of the experimental results were given in Tables 3 and 4.

Table 3. *In vitro* sensitivity of *N. kampachi* N-1 to various drugs by serial dilution method, at 25°C for 4 days.

Drugs	MIC value in mcg/ml
Oleandomycin	0.2
Dihydrostreptomycin	0.4
Chloramphenicol	1.6
Viomycin	6.3
Nitrofrazone	25
Oxytetracycline	50
Sulfamonomethoxine	50
Sulfamethomidine	50
Isonicotinic acid hydrazide	100

Table 4. *In vitro* senstivity of *N. kampachi* N-1 to drugs by resistance test method, at 25°C for 10 days.

Drugs	Conc. (mcg/ml)	Sensitivity
Kanamycin	0	−
	10	##
	100	##
Streptomycin	0	−
	10	##
	100	##
Sulfisoxazole	0	−
	1	+
	10	##
	100	##
Viomycin	0	−
	10	+
	100	##
Capreomycin	0	−
	10	−
	25	+
	100	##
Isonicotinic acid hydrazide	0	−
	0.1	−
	1	+
	5	+
Tibine	0	−
	1	−
	10	+
Para-aminosalicylic acid	0	−
	1	−
	10	−
Ethambutol	0	−
	2.5	−
	5	−
	10	−
Cycloserine	0	−
	10	−
	20	−
	40	+
Ethionamide	0	−
	12.5	−
	25	−
	50	−

##: high sensitivity, +: low sensitivity, −: no sensitivity

Experimental nocardiosis of yellowtails has been treated with these various antituberculosis drugs. The intramuscular inoculation or oral dosage of 4–100 mg/kg dose of drugs every day for 5–7 days proved to prolong the life-span of yellowtail infected with *N. kampachi*. The examples of the experimental results were given in Table 5.

Table 5. Therapeutic effect of antibacterial drugs to experimental nocardiosis of yellowtails.

Drugs	Days after treatment (mortality %)					
	3	6	9	15	21	28
Streptomycin						
1. Inoculation	0	0	0	40	40	40
2. Oral	0	0	0	0	100	
Oxytetracycline						
1. Inoculation	0	0	0	0	0	0
2. Oral	0	0	0	0	30	50
Viomycin						
1. Inoculation	0	0	0	0	0	0
2. Oral	0	0	0	50	70	70
Control	0	10	40	90	100	

No work has been carried out on the chemotherapy of nocardiosis in yellowtail, and consideration must be given to the relative practical and economic merits of treating cases in the marine fish farm. Whilist this is an extremely interesting field for research, in the meantime it seems that prophylaxis alone is the most acceptable way in which a prevent outbreaks of nocardial infection under the conditions peculiar to marine fish-farming.

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SPONTANEOUS DIABETES OF CARP, *CYPRINUS CARPIO*[1]

MOTOYOSHI YOKOTE[2]

In Japan, Sekoke disease has long been known to fish culturists and fisheries workers as one of the most puzzling diseases of carp. The affected fish are characterized by their skinny appearance, which extremely impairs the market value of themselves and sometimes results in a serious loss to the carp culturists.

For these 10 years, however, with a extensive improvement of fish diet, especially with replacement of silk-worm pupae by a pelleted fish-meal enriched with vitamines, the case tended to take a favourable turn.

Since 1960, the author has continued to study this disease, and a series of pathological and biochemical studies on the diseased carp have disclosed that they have diabetes mellitus with insulin-resistant hyperglycemia, a decreased glucose tolerance, glycosuria and ketonuria. Histopathology of their pancreatic islets, microvasculature, peripheral nerves, and muscular tissues are very similar to those of spontaneously diabetic animals so far described (Yokote, 1970a, b, c). This is the first report of spontaneous non-mammalian diabetes.

GROSS ANATOMY

The diseased carp showed a marked, skinny appearance, which was particularly distinct in the dorsolateral region of the body. Transverse section of the fish apparently indicated a general loss of flesh (Figs. 1 and 2). Shrinkage of fins was not uncommon finding. Occasionally, the disease was complicated by spinal curvature. The affected fish tended to show hypodermal hemorrhage in winter. In the visceral organs, no appreciable change was noticed with the naked eye except occasional hypertrophy of the kidney.

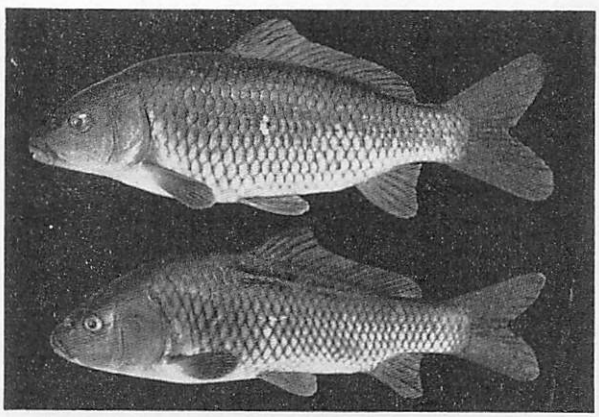

Figure 1. Sekoke carp (lower) and a normal control (upper). The fish are about 20cm in total length.

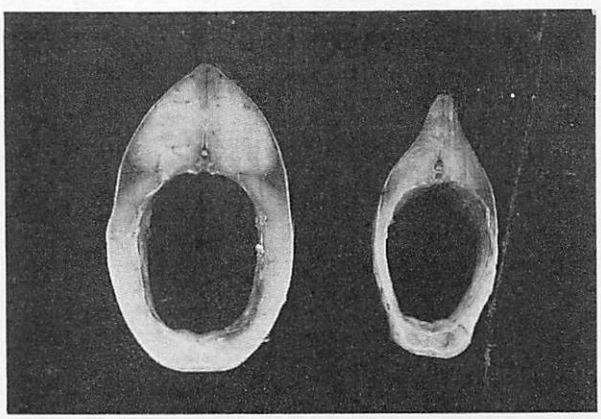

Figure 2. Transverse slices of the fish shown in Fig. 1. They were sectioned at the anterior end of the dorsal fin, respectively. Normal (on the left), Sekoke (on the right).

PATHOLOGICAL PHYSIOLOGY

1) Fasting Blood Sugar Level

Normal carp: Fasting blood sugar value for 106 normal controls was 39.2±0.8 mg/100 ml, ranging 25–54 mg/100 ml.

Sekoke carp: The diseased carp apparently showed hyperglycemia, at a fasting level of 77.6±3.6 mg/100 ml (n=61), ranging 42–194 mg/100 ml.

[1] Partly presented at 8th International Congress of Diabetes Federation (Brussels, 1973).

[2] Freshwater Fisheries Research Laboratory Hino-Shi, Tokyo, Japan

2) Glucose Tolerance

Normal carp: Blood sugar value for normal controls rose from a fasting level of 41 mg/100 ml to a peak of 127 mg/100 ml one hour after glucose loading (0.5 g/kg). The curve fell down to near normal level 5–6 hours after the treatment (Fig. 3).

Sekoke carp: Glucose tolerance curve of the diseased fish showed a delayed rise, higher attainment with a maximum case of 200 mg/100 ml, and tardy descent, a typical pattern representing decreased glucose tolerance (Fig. 3).

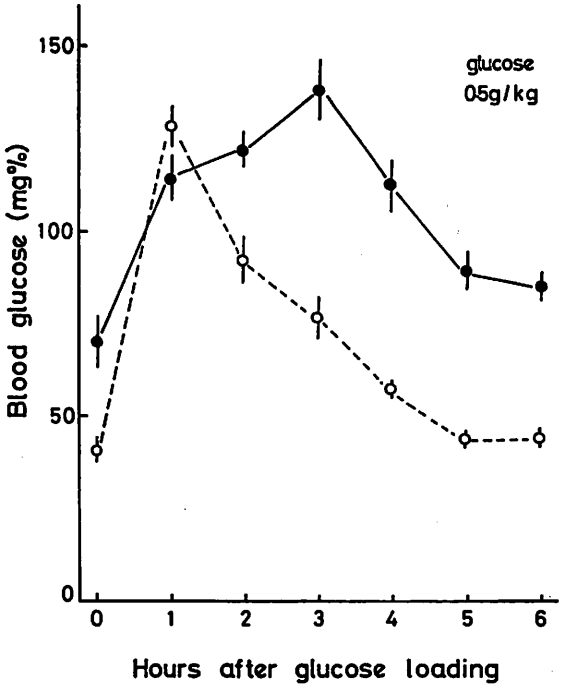

Figure 3. Glucose tolerance curve. Each dot (● Sekoke carp, ○ normal controls) with a vertical line expresses mean±SEM (n=10–22).

3) Urinalysis

Occurrence of abnormal urine was infrequent in 106 normal controls (8 cases with glycosuria of Light degree* including 3 suspected cases, and 2 ketonuria of Small degree**). On the contrary, glycosuria and ketonuria were often detected in Sekoke carp. Details are referred to Table 1.

4) Blood pH

Blood pH value was 7.5 on the average for 20 normal controls, ranging 7.4–7.6, whereas those for Sekoke carp had a wide range of variation (6.9–7.6, averaging 7.3). Furthermore, about a half of the latter were less than 7.4, the minimum value in the normal controls (Table 2).

Table 1. Occurrence of glycosuria and ketonuria in Sekoke carp.

	Glycosuria (74 animals)	Ketonuria (50 animals)
Negative	6	13
Light[1] (Small[2])	53	34
Medium[1] (Moderate[2])	13	2
Dark[1] (Large[2])	2	1

[1] Standard for Clinistix
[2] for Ketostix

Table 2. Blood pH for Sekoke and normal carp.

	Number of specimen	pH value							
		6.9	7.0	7.1	7.2	7.3	7.4	7.5	7.6
Normal carp	20	0	0	0	0	0	2	10	8
Sekoke carp	28	1	0	3	2	7	3	6	6

5) Response to Mammalian Insulin

Normal carp: As shown in Fig. 4, 10 IU/kg of crystalline bovine insulin produced a marked reduction of blood sugar 5 hours after insulin injection with a return to near normal level by 20–24 hours.

Sekoke carp: The tolerance curve of the diseased fish apparently indicated a refractoriness to the hormone. The mode of the curve characterized with a decreased lowering rate, tardy fall, and delayed attainement to the lowest level is alike to those generally referred to as 'insulin-resistant' in diabetic animals so far reported (Fig. 4).

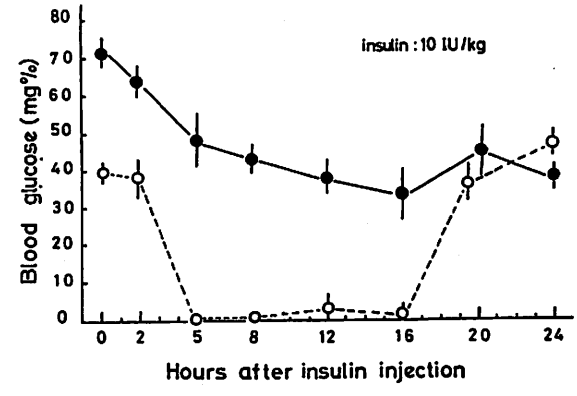

Figure 4. Effects of intramuscularly injected insulin on the glycemic levels of Sekoke and normal carp. Each dot (● Sekoke carp, ○ normal controls) with a vertical line expresses mean±SEM (n=8–18).

* Standard for Clinistix,
** for Ketostix

HISTOPATHOLOGY

1) Endocrine Pancreatic Tissues

Histological structure of endocrine pancreatic tissue of fishes has been described so far by various workers. In particular, morphology and functional aspects of the islet tissue have increasingly come to light with the progress of electron microscope. Today, two major types of islet cell, the A and B have been recognized in many animals including fishes. However, it is still controversial as for the nature of the third or fourth type of cells.

Pancreatic islets of carp consist of several isolated small knots, so-called Blockmann's body, 1–2 mm in diameter, milky-white in color, being easily found with the naked eye behind the gall-bladder. Besides these principal islets, many smaller ones are observed to be embedded in the fatty tissue around the intestinal tube. Microscopically, almost all of smaller islets are sorrounded by exocrine pancreatic tissue which sometimes runs into the liver. But, pancreatic islets are always demarcated by a thin capsule of connective tissue. The normal islet tissue consists of four types of cells, the A, B, D and a small number of the

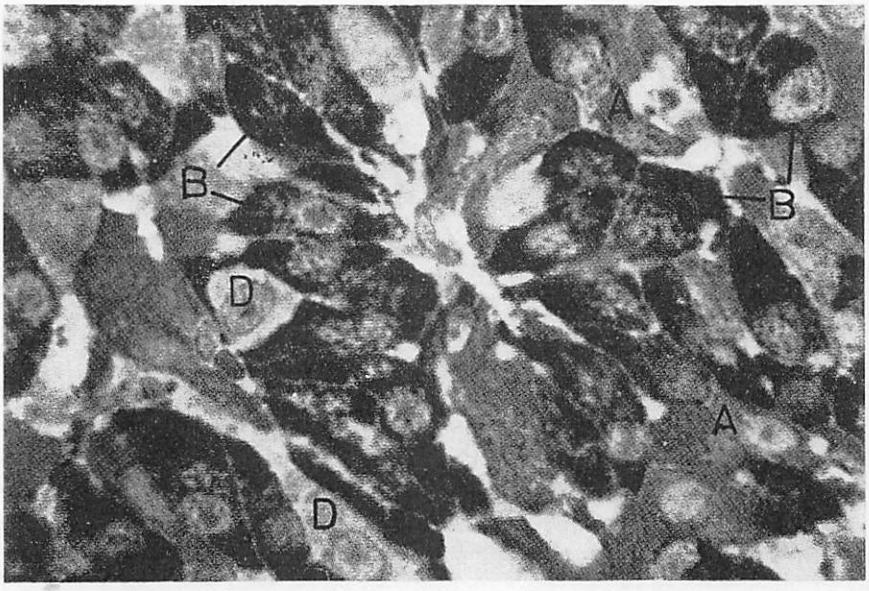

Figure 5. Normal pancreatic islet. AT-azocarmine G stain, ×1000

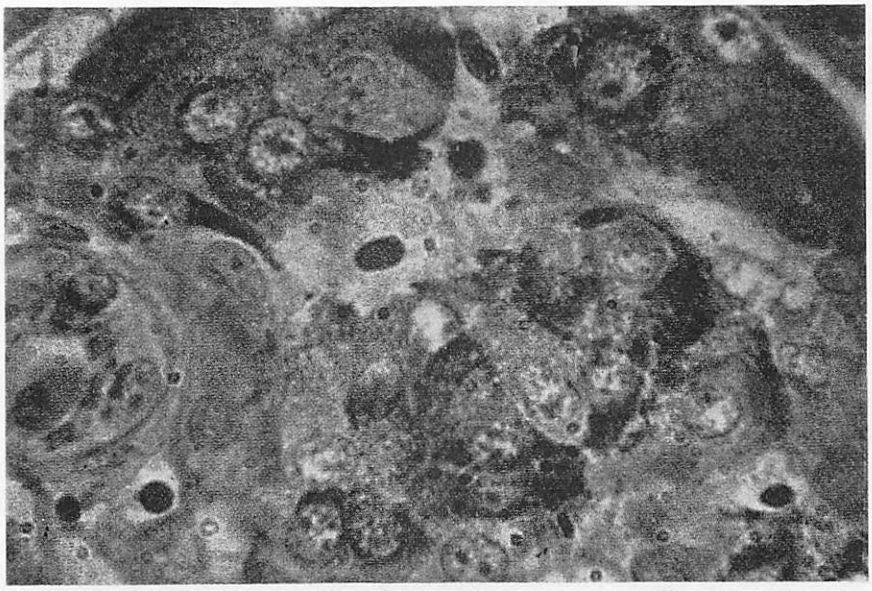

Figure 6. An islet from a Sekoke carp showing the B cell degranulation and nuclear hypertrophy. AT-azocarmine G stain, ×1000

clear cell (Yokote, 1970a). Recently, Nakamura and Yokote (1971) reported that the clear cell is a variety of the B cell.

Microscopical change of the pancreatic islets was one of the most characteristic findings in Sekoke carp. Complete or partial degranulation of the B cells was frequently observed. In these states, the B cells often showed nuclear hypertrophy with obscure cell contour, loss of uniformity in nuclear size, and occasional mitotic division (Figs. 5 & 6). Sometimes, cytoplasm of the B cells showed empty-looking or moth-eaten appearance with pyknotic nuclei. A large amount of glycogen was clearly demonstrated in the corresponding loci by the PAS method. Nakamura et al. (1971) confirmed on the ultrastructural basis that the pancreatic islets of Sekoke carp are characterized by the presence of appreciably more numerous clear cells than those in the analogous glands of normal carp. Within the cytoplasm of the clear cells of the diseased carp, a large number of tiny vesicles with a diameter comparable to that of Golgi vescicles are disseminated throughout. In addition, the elements of granular endoplasmic reticulum in these cells are rather well developed and often represent lamellar arrangements of the cisternae. These morphological features imply that there is enhanced B cell hormone synthesis and release in the insular tissues of the diseased carp.

2) The Kidney

Pathological process observed in the glomerulus of Sekoke carp was highly characteristic and closely similar to diabetic renal lesions so far described for man and other animals.

In most cases, glomerular capillaries were extremely distended, with or sometimes without PAS-positive thickening of capillary wall. Distended capillaries often left no space of Bowman's capsule. Diffuse thickening of the mesangial stalks was characteristically observed in almost all cases without any definite nodules such as described by Kimmelstiel and Wilson (1936). Accumulation of amorphous substances was conspicuously observed in the mesangium with the PAS method (Figs. 7 & 8). These substances, being resistant to digenstion with amylase, appeared to be closely related to the PAS-positive materials in the capillary wall. Occasionally, acellular exudates with strongly PAS-positive property was found in the space of Bowman's capsule. At times, thickening of the capsular basement membrane and proliferation of capsular epithelium were observed.

In relation to these alterations, hyalinosis of afferent and efferent arterioles and related smaller blood vessels was encountered.

3) The Eye

Involvement of microvasculature in the retinal region and choroidal vessels was very characteristic for Sekoke carp and the alteration was essentially similar to diabetic retinopathy of man and other animals.

Macroscopically, extremely tortuous and dilated vitreous vessels were clearly noticed over the retina after the removal of the front half of the eye ball.

Microscopically, capillary lumen was extremely distended with or without PAS-positive thickening of the vessel wall and proliferation of endothelial

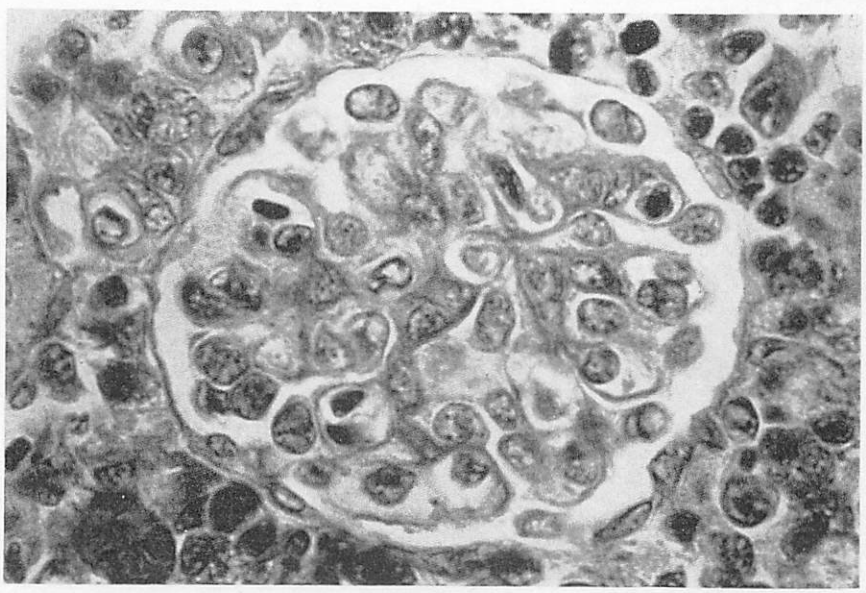

Figure 7. A glomerulus of the kidney from a normal carp. PAS stain, ×600

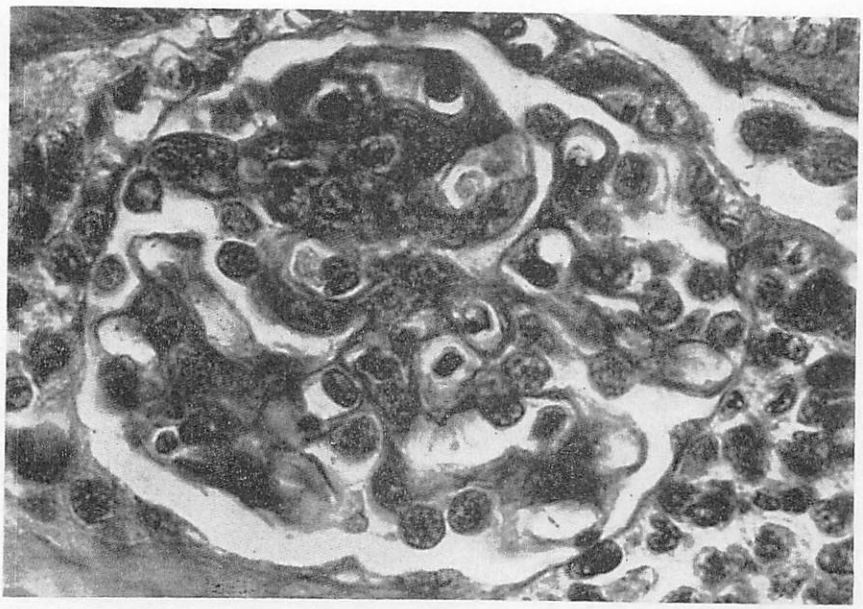

Figure 8. A glomerulus of the kidney from a Sekoke cap. Note PAS-positive thickening of capillary wall and mesangial region. PAS stain, ×600

cells. Sometimes, cut ends of dilated capillaries were looked like a string of beads along vitreo-retinal boundary (Figs. 9 & 10). Occasionally, proliferation of small round cells of uncertain identity was observed in a part of the inner nuclear layer with extention into the adjacent layers. These alterations resulted in a protrusion of a part of retinal parenchyma into the vitreous cavity. In most cases, choroidal capillary showed PAS-positive thickening of the vessel wall. Dilated and tortuous capillaries were best visualized on the flat preparation by trypsin digestion. Dilated capillaries often showed peculiar protrusions on the vessel wall at indefinite points. These 'capillary buds' were small outpouchings of a vessel wall in some specimens. A slightly larger protuberances with more-or-less PAS-positive property were found in others. The alteration was most frequently observed in the plexus of capillaries. Exact counterparts of microaneurysm and totally acellular capillary described for man and other animals were not detected.

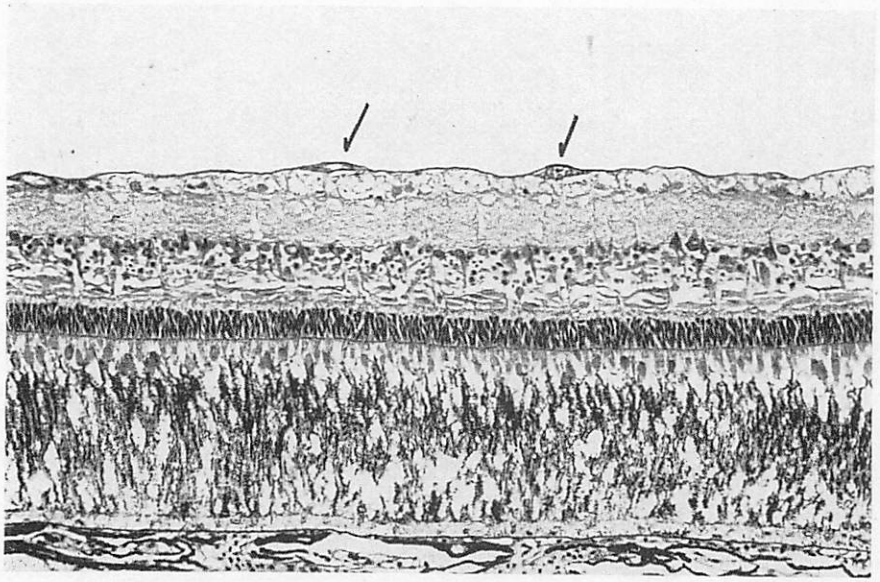

Figure 9. Retina from a normal carp. Arrows indicate vitreous capillaries. PAS stain, ×150

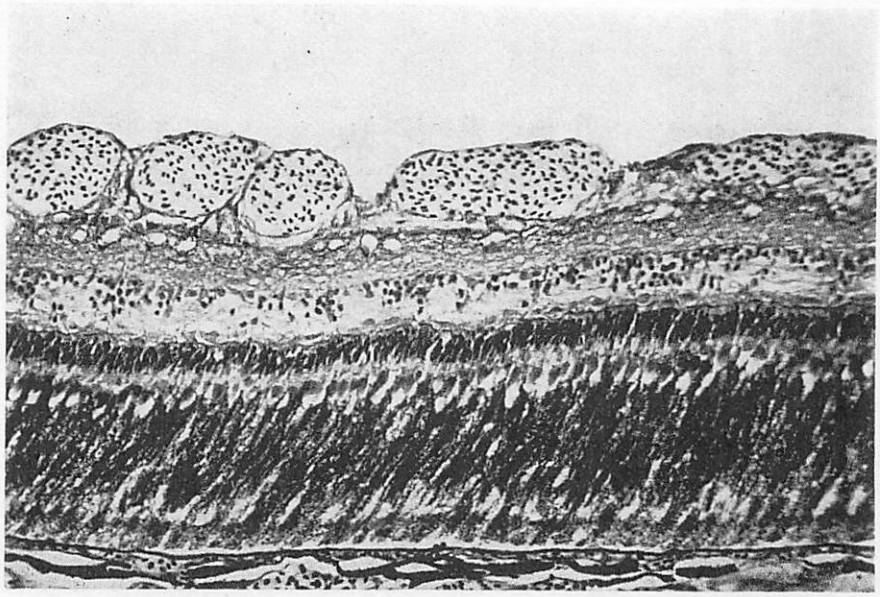

Figure 10. Retina from a Sekoke carp showing extremely dilated capillaries. PAS stain, ×150

4) The Gill

The bonny fish has the gill, a respiratory apparatus, having a well-developed vascularization. The gill of Sekoke carp often showed capillaropathy which is very similar to those of retina and glomerulus in their nature. Thickening of basement membrane and dilatation of gill capillary were often detected in the diseased carp. These microvascular changes including retinopathy and glomerulopathy suggest the existence of generalized microvascular change in the diseased carp (Fig. 11).

5) Striated Muscle

Histological change of the skeletal muscle was a striking feature in Sekoke disease. Presumably, these muscle changes might result in the skinny appearance, or at least its part, of the affected fish. Although the muscle change varied with fish and from muscle to muscle, the alteration was characterized with wide-spread degeneration (Fig. 12). The lesions involved not only ordinary muscle, but also red one,

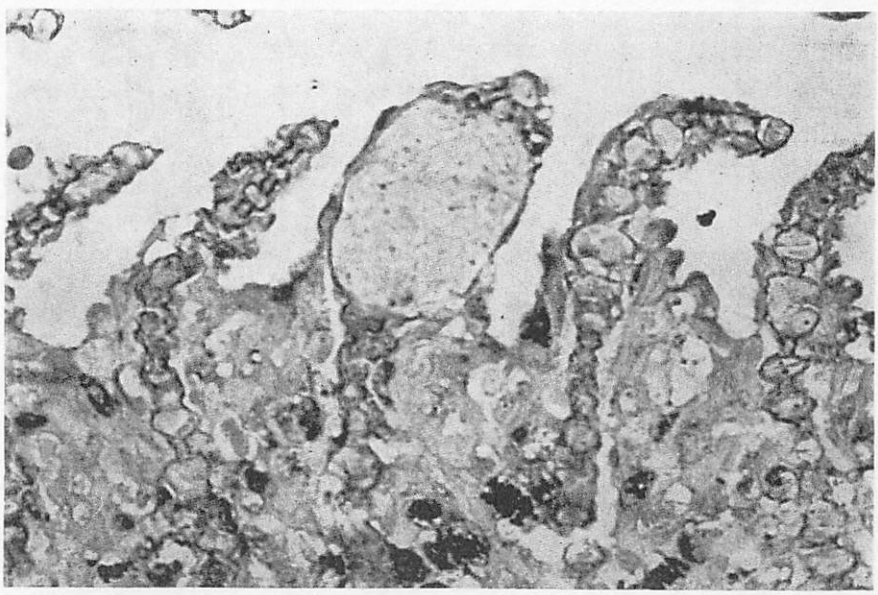

Figure 11. A part of gill filament from a Sekoke carp. Dilated capillary is seen in the center. Alcian blue-PAS stain, ×600

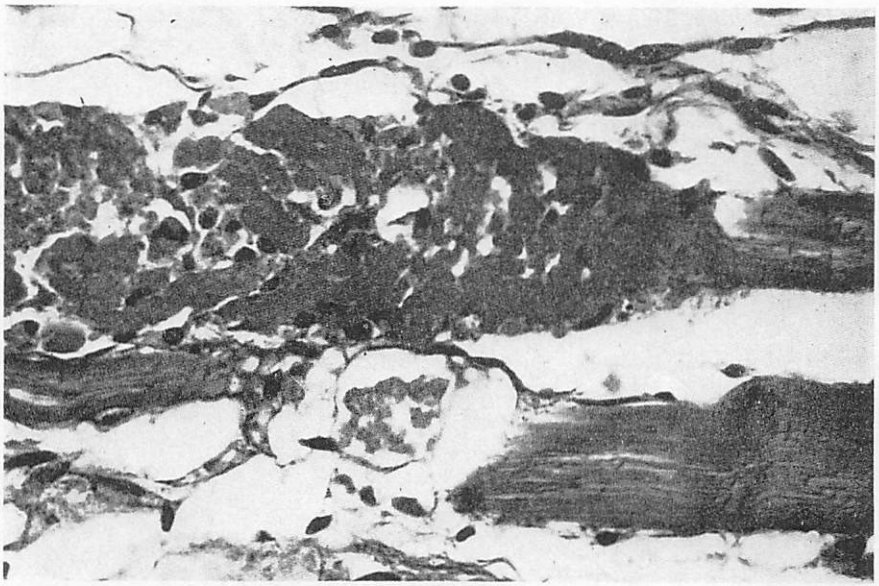

Figure 12. Granular degeneration of muscle fiber seen in the skeletal muscle of Sekoke carp. H-E stain, ×400

and a predilection for involvement of a particular muscle was not found. A swelling of all or a segment of muscle fiber with more-or-less cloudy appearance was one of the earliest changes, and the alteration was followed by hyaline degeneration and granular necrosis. Terminal phase of granular degeneration was initiated with phagocytic invasion indicating removal of necrotic materials, and regeneration of muscle fiber began to occur gradually. In this period, thin and short fibers with distinct transverse striation were often observed. It should be mentioned that the regenerated fibers were smaller in bulk than those of normal controls even after the completion of new fasciculata.

As associated findings, demyelination and dilatation of axon were frequently observed in the ventral branches of the ventral root. Intermuscular nerves arising from the branches were also involved (Fig. 13). In most cases, the lateral line nerve, a kind of sensory nerves, of the diseased carp showed pathological changes such as demyelination, thickening of the sheath, and dilatation of the axon. The relation

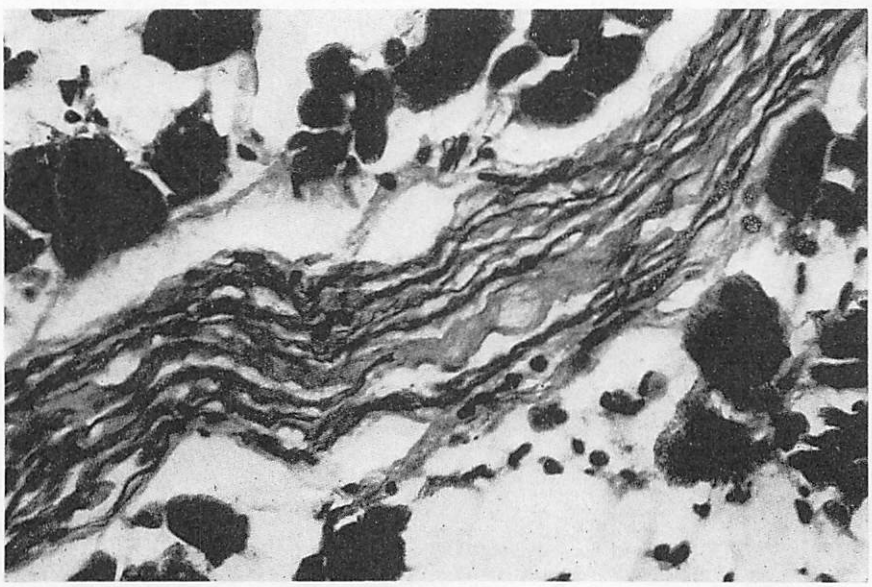

Figure 13. Intermuscular nerve bundle of Sekoke carp showing dilated and distorted axons. Holmes' silver impregnation, and Luxol fast blue MBS myelin stain, ×600

between these neural changes and muscle degeneration is not clear.

6) Others

Some histological changes were observed in the liver, adrenocortical tissue, meso-adenohypophysis, thyroid gland, exocrine pancreatic tissue, and gonads of the diseased carp. Since the occurrence of these alterations was inconsistent, they are conceived to be secondary to a generalized metabolic derangement.

COMMENT

In recent years, spontaneous diabetes in animals, especially in rodents such as spiny mouse, sand rat, Chinese hamster, and some imbred strains of mouse has attracted many worker's attention in the diabetes research. In fishes, however, spontaneous diabetes has not been reported so far. In this connection, it should be emphasized that Sekoke disease found in carp is the case of spontaneous diabetes mellitus. At the same time, histological findings obtained in the present study, especially microangiopathy, myopathy and neuropathy, are very interesting in view of fish pathology as well as comparative diabetology.

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SOME POTENTIALLY SERIOUS DISEASE PROBLEMS IN THE CULTURE OF PENAEID SHRIMP IN NORTH AMERICA[1]

DONALD V. LIGHTNER[2]

INTRDUCTION

Successful commercial culture of shrimp is not yet a reality in North America. The ever-increasing demand for shrimp coupled with rising prices for shrimp has lead to increased interest in the development of commercially viable shrimp farms (Neal, 1973a). Shrimp farms using the traditional pond culture approach on a pilot scale are located in Florida, Louisiana, and Texas. One promising approach to shrimp culture in North America is raceway culture (Neal, 1973b). This method is being studied in Galveston, Texas, and in Puerto Peñascol Sonora, Mexico.

Among the factors delaying successful development of shrimp culture in North America is the need for the development of methods for the diagnosis, treatment, and prevention of disease. At the present time there are at least five major disease of penaeid shrimp in North America that are likely to pose obstacles to successful commercial culture. These five disease are: (1) a mycosis of larval shrimp caused by a *Lagenidium* sp.; (2) mycotic infection of juvenile shrimp with *Fusarium* spp.; (3) bacterial infections caused by *Vibrio* spp. and *Beneckea* spp.; (4) a complex of several gill diseases the causes of which individually or collectively result in respiratory failure; and (5) the "cotton shrimp" group of diseases caused by several species of microsporidia.

MATERIALS AND METHODS

Most of the shrimp for these studies were obtained from the National Marine Fisheries Service shrimp hatchery and rearing facility at Galveston, Texas or from the University of Arizona-University of Sonora experimental shrimp farms at Tucson, Arizona and Puerto Peñasco, Sonora, Mexico. Additional shrimp were obtained from the Dow Chemical Company experimental shrimp hatchery and rearing ponds at Freeport, Texas, and from the Texas Parks and Wildlife Department shrimp rearing ponds at Palacios, Texas. Some wild shrimp from commercial bait dealers on Galveston Bay were also used.

Methods of diagnosis and isolation, culture, and identification of presumed pathogens are given in the appropriate sections for each of the diseases discussed.

Shrimp selected for histological examination were fixed live in either 10% phosphate buffered formalin, Carnoy's fixative or Davidson's fixative. In the case of small shrimp (under 60mm in total length) the cuticle over the hepatopancreas and over the abdominal musculature and midgut was opened with scissors to enhance fixative penetration. In the case of larger shrimp, body regions that contained the organs or tissues of interest (the gills for example) were removed and fixed separately. Embedding, sectioning, and staining were accomplished using routine histological methods.

RESULTS AND DISCUSSION

Larval Mycosis

The occurrence of a *Lagenidium* sp. in penaeid shrimp was first observed in the spring of 1971 at the Dow Chemical Company's shrimp hatchery at Freeport, Texas (Cook, 1971). The fungus caused extensive mortality of brown shrimp (*Penaeus aztecus*) larvae within 2 to 3 days in hatchery tanks. The disease appeared sporadically during the remainder of 1971 and again in 1972 and 1973. Each time it appeared it resulted in high mortalities, sometimes nearly 100% (personal communication, Bruce Hysmith, present address: Texas Parks and Wildlife Department, Palacios, Texas).

In the summer of 1972 a fungus disease of larval white shrimp (*P. setiferus*) caused by a *Lagenidium* sp. occurred at the National Marine Fisheries Service shrimp hatchery in Galveston, Texas (Lightner and Fontaine, 1973). The presence of the disease first became apparent when the white shrimp larvae reached the second protozoeal stage. The infection

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was limited to one hatchery tank and mortality due to the disease was 12%. Mortality stopped as the remaining larvae reached the first mysis stage. Despite the presence of large numbers of unencyted zoospores in the tank water at the time, none of the mysis stage larvae examined from several random samples were infected by the fungus.

Lagenidium disease of larval penaeids has also been reported from other shrimp hatcheries. Barkate, et al. (In Press) reported that the disease had occurred at the Ralston Purina hatchery at Crystal River, Florida. There the disease was responsible for complete mortality in one hatchery tank within 2 days after its appearance. On other occasions the disease

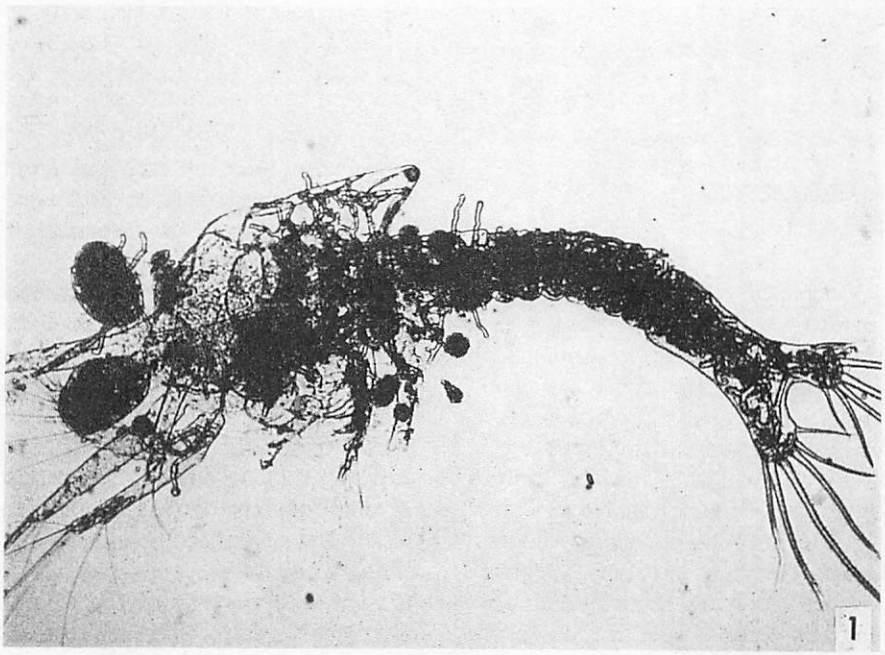

Figure 1. Larval white shrimp (protozoea II) heavily infected with a *Lagenidium* sp. Extramatrical hyphae, some with terminal vesicles, are shown protruding from the shrimp. No stain. ×72.

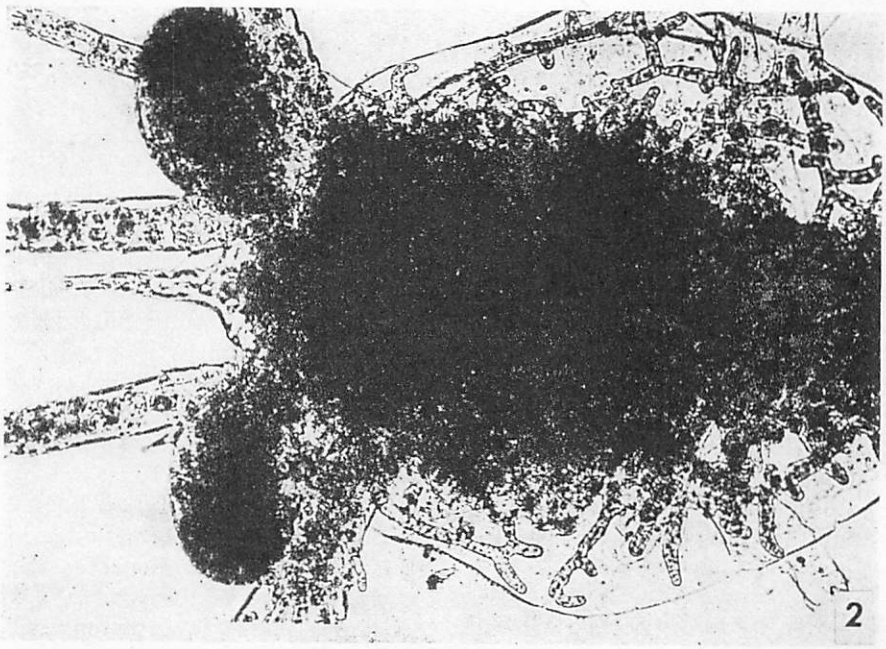

Figure 2. Larval white shrimp with hyphae of *Lagenidium* sp. occupying much of the space in the cephalothorax, eye stalks, and appendages. No stain. ×140.

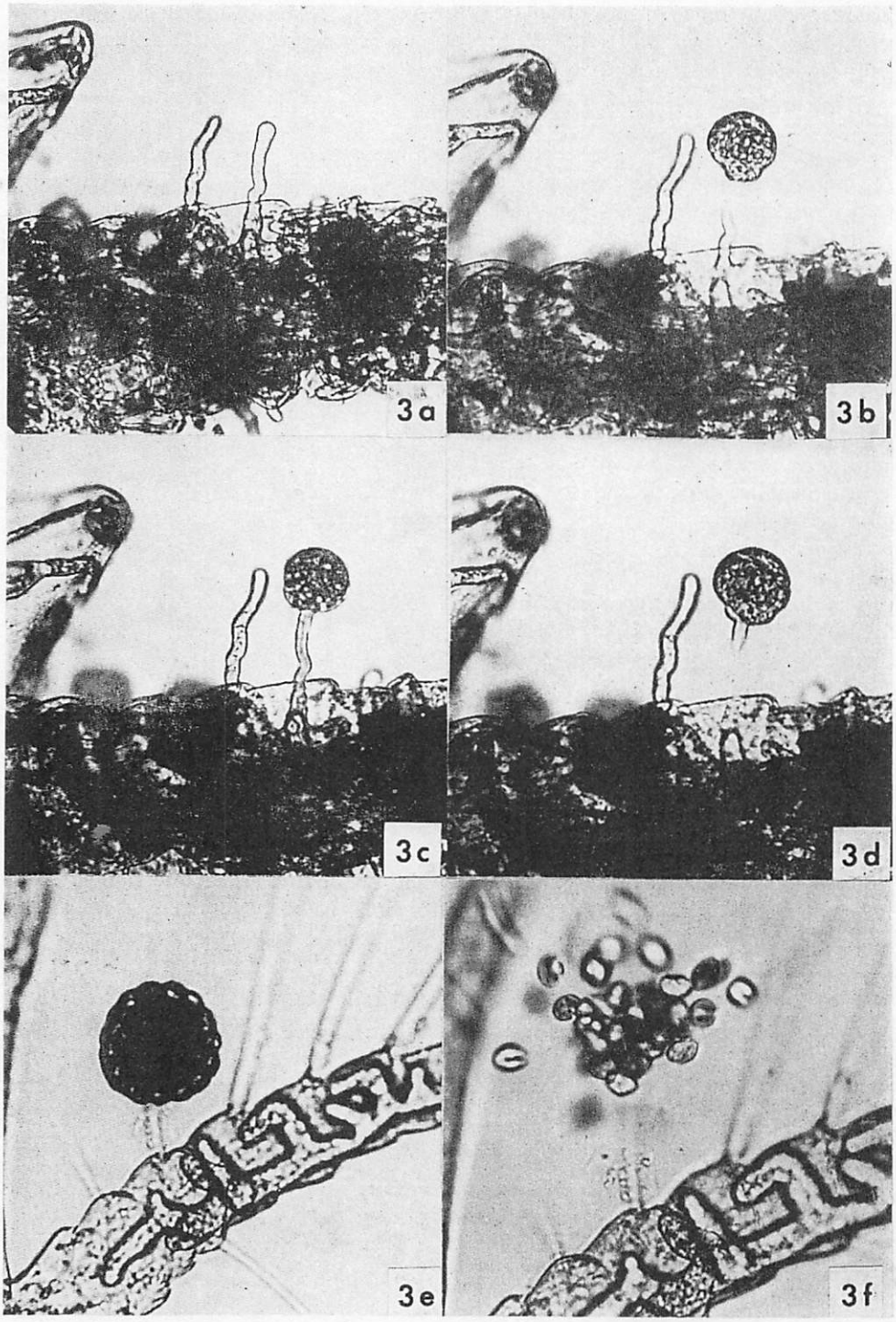

Figure 3. (a) An extramatrical "discharge tube" from the same shrimp as shown in Fig. 1. No Stain. ×220; (b, c, d) A unit of cytoplasm is shown flowing through the "discharge tube" into the vesicle. No Stain. ×252; (e) A vesicle in which the outline of individual planonts (zoospores) has become apparent. No Stain. ×315; (f) The same vesicle as in 3c during planont discharge. The planonts are reniform and are motile by two flagella which arise from the lateral groove. No stain. ×800.

was observed, but it was not always responsible for extensive mortalities. Barkate did no indicate what species of penaeids were involved in the epizootics at Crystal River.

Brown shrimp (*P. aztecus*) larvae were found to be highly susceptible to the fungus when exposed experimentally as protozoea stage I or stage II larvae. In one experiment in which 2,000 stage I protozoeal larvae were exposed to zoospores and hyphae of the fungus, mortality reached 20% by 96 hours after inoculation. In a more recent experiment in which 4,000 stage I and stage II protozoeal brown shrimp were exposed to zoospores of *Lagenidium*, a 97% mortality occurred by 96 hours after inoculation. Infection of mysis I larvae was observed in one latter experiment.

In natural and experimental epizootics in brown and white shrimp larvae, the protozoeal stages seemed to be the most susceptible. Occasionally infected larvae in the last naupliar stage or the first mysis stage were observed, but infection of the protozoeal stages was most typical.

The earliest sign of infection in a larval white or brown shrimp was the presence of hyphae within the appendages. Such individuals were much less active than noninfected controls. Infection occurred when a zoospore encysted upon a susceptible larva and germinated. If the germinal hyphae penetrated the cuticle, an infection was established. The fungal mycelium gradually invaded and replaced nearly all of the striated muscle tissue of the larval shrimp (Fig. 1). The thorax, abdomen, swimming appendages, and even the eye stalks became filled with hyphae (Figs. 1 and 2). Massive tissue destruction, particularly of the striated muscle, resulted in immobilization of the shrimp as much as 1 hour before death. An occasional movement of an appendage or contraction of the hindgut musculature were the only signs of life seen in these shrimp.

Soon after death of an infected larvae, the process of sporulation began with the emergence of "discharge tubes". The apical end of these "discharge tubes" swelled, forming a vesicle as it filled with individual units of cytoplasm which flowed from a sporangium located on an intramatrical hypha located within the body of the larva (Fig. 3). Planonts or zoospores developed from the amorphous mass of cytoplasm in the vesicle and were released when their movements ruptured the vesicle. Planonts were reniform, motile by two flagella that originated from the lateral grove, and were 8.7 by 12.0μm in size (Fig. 3).

Lagenidium sp. from penaeids was easily isolated and cultured in several types of media. Isolation was facilitated by the addition of penicillin (500 units/ml of medium) and streptmycin (500μg/ml of medium) to isolation medium to inhibit bacterial growth. For inoculation of isolation media, a single infected larva or a few milliliters of the seawater containing infected larvae and planonts was introduced into isolation medium. Best growth was obtained on Sabouraud dextrose agar enriched with 2% NaCl and shrimp homogenate. On this medium growth of the fungus was rapid and the mycelium typically had covered the entire agar surface of a 100mm plate by 4–5 days after inoculation and incubation at 28°C (Fig. 4).

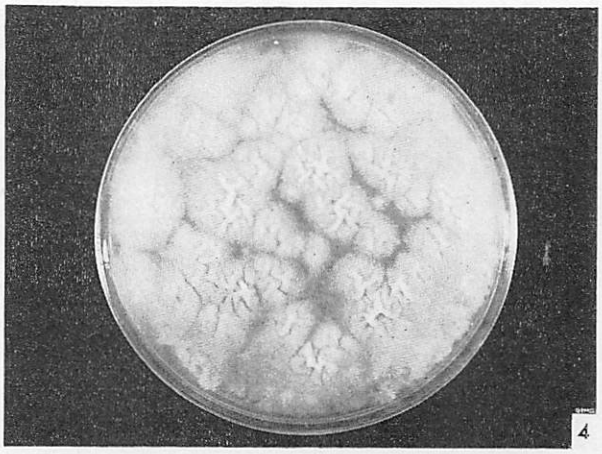

Figure 4. A 5-day-old culture of the *Lagenidium* sp. from the white shrimp (*P. setiferus.*). Culture medium was Sabouraud dextrose agar enriched with 2% NaCl and shrimp homogenate. Incubation temperature was 28°C.

Means of control or treatment of *Lagenidium* infections in larval penaeids are not known and, as yet, the method by which the fungus is introduced into shrimp hatchery tanks has not been determined. Cultures made of ovary homogenates from spawning female brown shrimp have been negative for *Lagenidium* even when the larvae obtained from these females later developed the disease. A closely related species, *Lagenidium callinectes*, has been reported from the eggs and larvae of the blue crab, *Callinectes sapidus*, (Couch, 1942; Rogers-Talbert, 1948) and from the eggs of a barnacle (*Chelonibia patula*) that occurs commonly on the carapace of blue crabs and other marine animals (Johnson and Bonner, 1960). Another closely related species *L. chthamalophilum* has been reported as a parasite of the ova of the barnacle *Chthamalus fragilis* (Johnson, 1958). It is possible that various species of crabs and barnacles that occur naturally in the water source for shirimp hatcheries carry the *Lagenidium* sp. that is pathogenic to penaeid shrimp larvae as a normal parasite of their egg masses and hence serve as a reservoir for the parasite.

To date, all treatments of affected larvae tested

have been unsuccessful. All of the promising broad-spectrum antibiotics and fungicides tested have been determined to be toxic to penaeid shrimp lavae at the concentrations needed to kill or inhibit growth of the fungus. One chemical which shows promise in controlling the fungus is malachite green oxalate. Malachite green oxalate concentrations of 0.01 to 0.06 ppm (Bland, in press) was found to be effective in inhibiting the growth of *Lagenidium* sp. from white shrimp, but its toxicity to larval penaeids has not been tested.

Fusarium Disease

Imperfect fungi belonging to the genus *Fusarium* have been reported from the Kuruma prawn, *Penaeus japonicus*, in Japan (Egusa and Ueda, 1972) and from laboratory-held pink shrimp, *P. duorarum*, in Texas (Johnson, 1974b). An additional *Fusarium* sp. has been described from the lobster, *Homarus americanus*, from a small experimental lobster farm in New York (Lightner, in press; Lightner and Fontaine, in press).

The *Fusarium* sp. in *P. japonicus* seemed to be a new species and the fungus was designated BG-Fusarium (Black Gill Fusarium) until it could be named (Egusa and Ueda, 1972). As the name "Black Gill Fusarium" implies, the fungus causes a disease that produces black gills in *P. japonicus*. The disease was shown to be the cause of serious mortalities among pond-cultured prawn populations. Affected parts of the gills carried septate hyphae of the fungus. Intramuscular inoculation of healthy prawns with conidia of the fungus caused "black gill disease", and the fungus was isolated from gill lesions of artificially infected prawns. On may culture media, including Sabouraud's dextrose agar medium, the fungus produced a dark purplish brown diffusable pigment.

The *Fusarium* sp. in pink shrimp (*P. duorarum*) did not cause a black gill condition but did infect the gills and the antennal scales. Less than 5% of the laboratory population were found to have the disease and in these the spread of the fungus was slow, taking up to 2 weeks to develop into more than 10% of the body area (Johnson, 1974b).

Lobsters (*H. americanus*), infected with a *Fusarium* sp. nearly identical morphologically to BG Fusarium (Lightner and Fontaine, in press), developed focal melanized cuticular lesions on the exoskeleton, appendages, and gills. A generalized "black gill" condition was not observed, although death of affected lobsters seemed to result from rapid antemortem growth of the fungus in the gills and consequent respiratory failure.

Cook (1971) reported an unidentified species of fungus that infected juvenile brown shrimp (*P. aztecus*) in the hatchery. Lesions due to the fungus appeared as black spots and proved fatal when they spread to the gill region. This fungus may have been a *Fusarium* sp.

Another *Fusarium* sp. that differs morphologically from the *Fusarium* spp. of *P. japonicus*, *H. americaus*, or *P. duorarum* was shown to be the cause of a severe epizootic in the California brown shrimp (*P. californiensis*) in June and July of 1974, at the University of Arizona's experimental shrimp farm at Puerto Peñasco, Mexico. The disease was confined two raceways at the farm, but in one of the raceways nearly 100% incidence of infection was observed. Of the arproximately 6,000 100mm shrimp present in this raceway when the disease first became apparent, only 600 survived to July 12. The fungus, a *Fusarium* sp., typically infected the gills, the coxal (basal) segments of the walking legs, and the body wall behind the gills and above the coxal segments. The coxal segment, gill process, and adjacent portions of the 14th segment (the segment having the last or 5th walking legs) were nearly always infected by the fungus (Fig. 5). Other areas, such as the gill cover and the ventrolateral portions of the first abdominal segment, as

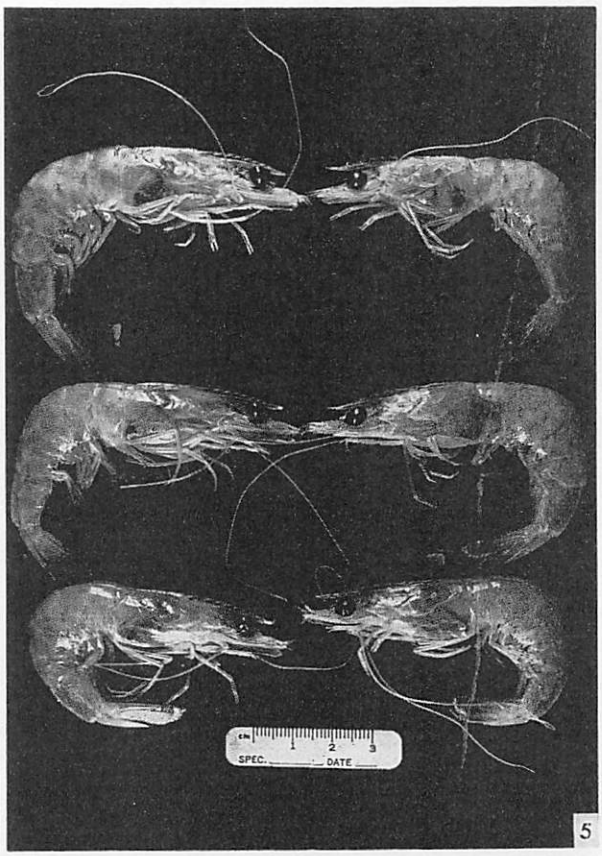

Figure 5. California brown shrimp (*P. californiensis*) infected with a *Fusarium* sp. Note the blackened gills, particularly above the 4th and 5th pereiopods.

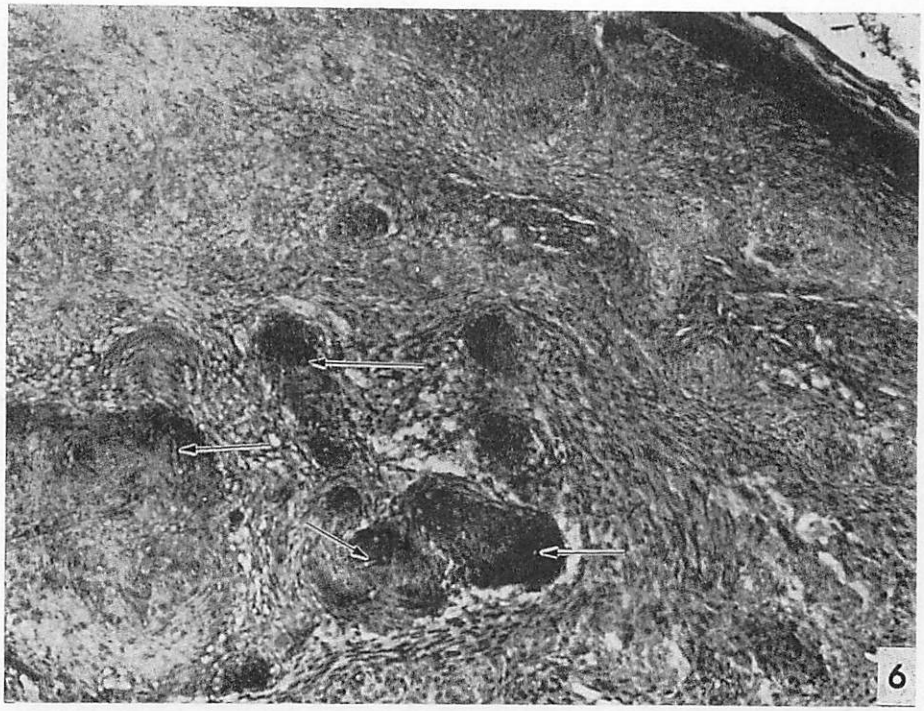

Figure 6. Photomicrograph of a histological section through a melanized *Fusarium* lesion in the body wall above the 5th pereiopod and gill process. The dark band in the upper right corner is melanin. Masses of hemocytes are encapsulating hyphae (arrows) of the *Fusarium* sp. Some of these cncapsulations are melanized near their centers. Hematoxylin and eosin. ×250.

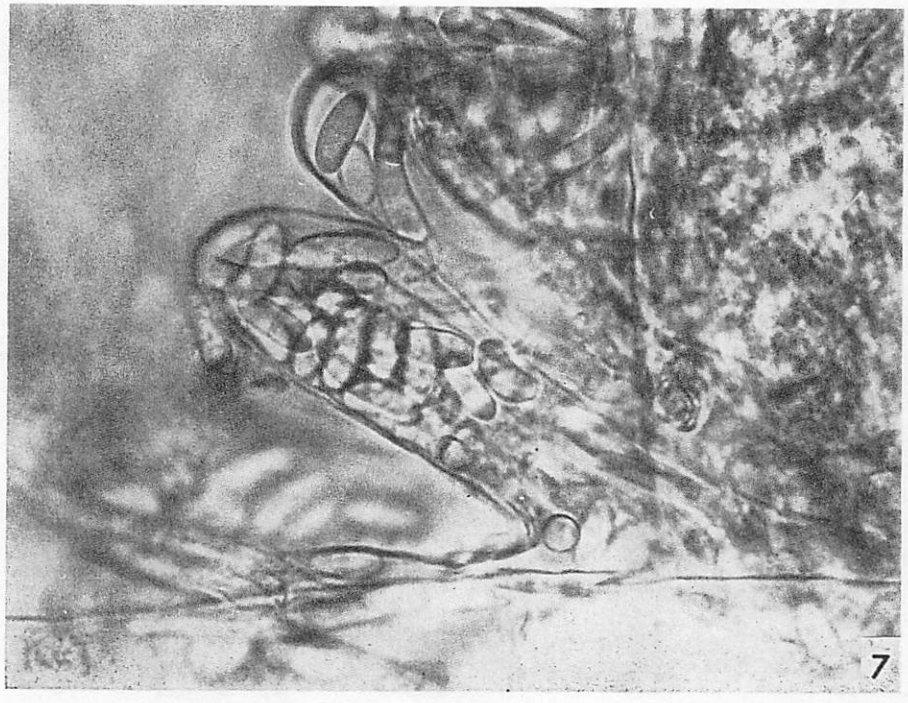

Figure 7. Photomicrograph of a wet mount of the gills of *P. californiensis* that died due to destruction of the gills by a *Fusarium* sp. Hyphae, microconidia, and macroconidia, and macroconidia are visible within the gill lamellae. No stain. ×840.

well as wounds at various locations on the shrimp, were occasionally found to be infected by the fungus. In every instance, at least some of the lesions due to the fungus on a particular shrimp, were marked black by deposition of melanin. Hence, most of the affected shrimp showed at least a limited "black gill" condition (Fig. 5). In these black lesions (Fig. 6) the melanin deposition resulted from the activity of hemocytes responding to the presence of hyphae and to tissue destruction caused by the fungus. Encapsulation of hyphae was typical when hyphae were present in subcutaneous or muscle tissues. Death in affected shrimp, as with *Fusarium* infections in the Kuruma prawn and the lobster, probably resulted from destruction of the gills by a rapid antemortem growth of the fungus into the gill processes that was not accompanied by an appreciable hemocyte response (Fig. 7).

The fungus was isolated in pure culture from gill lesions of every shrimp sampled that had lesions like those described above. Isolation media were Sabouraud dextrose agar supplemented with 2% NaCl and shrimp homogenate (SSS medium) and Cantino PYG both supplemented with 2% NaCl. Penicillin and streptomycin were added to isolation media to inhibit bacterial growth. Large numbers of macroconidia were produced by this *Fusarium* sp. on SSS media. From a single 100mm diameter SSS plate after 10 days of incubation at 28°C, 5.8×10^9 macroconidia were recovered from saline washings of the agar surface.

The *Fusarium* sp. from *P. californiensis* produced micro and macroconidia in artificial media and in shrimp tissues. Microconidia were typically ovoid to oblong and frequently slightly curved (Fig. 8); they were one-celled, or two-celled, and ranged from 9 to 18μm in length. Macroconidia were typically three-celled or four-celled and canoe shaped, or occasionally, cigar shaped. Macroconidia ranged in length form 30 to 47μm (Fig. 9). The fungus produced a pale brown diffusable pigment on SSS medium. This pigment is much paler than the dark purplish brown pigment produced by the *Fusarium* sp. from *P. japonicus* (Egusa and Ueda, 1972)

The probable source of conidia that infected the shrimp was determined. At Puerto Peñasco sets of two raceways were enclosed in a single air-inflaeted plastic greenhouse. Water flowed through the raceways and drained into a common sump at the end of the greenhouse. The blower that inflated the greenhouse was located in the outside wall of the sump. Hence, air currents carried spray from the sump over the raceways where much of the spray settled. The *Fusarium* sp. was cultured from water and debris in the sump, from spray in the sump, and from the air above the raceways. SSS plates exposed to the air

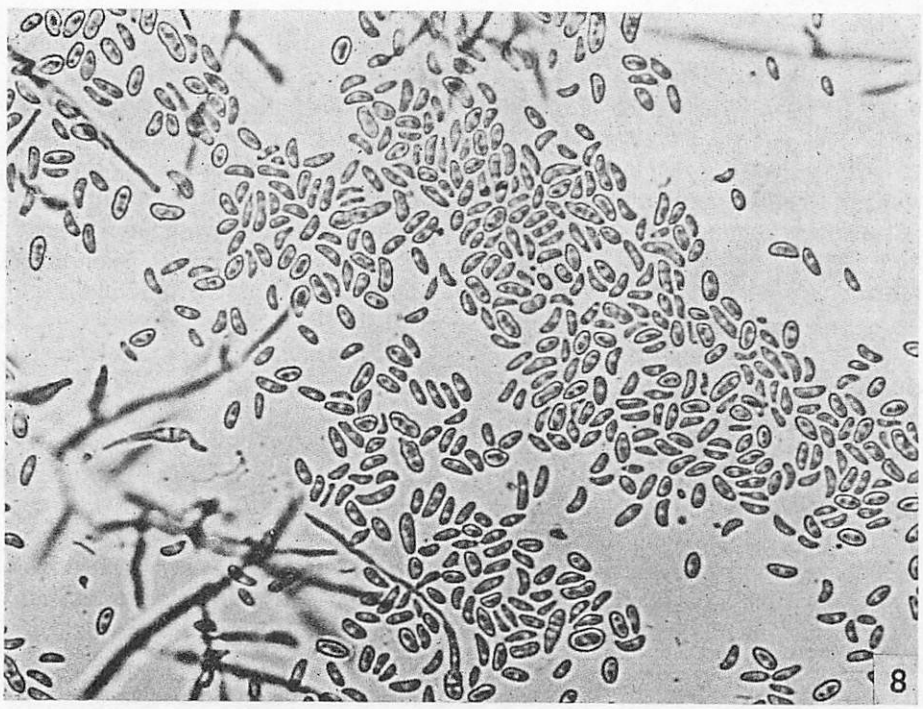

Figure 8. Photomicrograph of microconidia of the *Fusarium* sp. from *P. californiensis*. From PYG broth culture. No stain. ×300.

Figure 9. Photomicrograph of macroconidia of the *Fusarium* sp. from *P. californiensis*. From Sabouraud dextrose agar enriched with 2% NaCl and shrimp homogenate. No stain. ×500.

in the greenhous for only 10 minutes had colonies of the *Fusarium* sp. after 48 hours incubation at 28°C.

No effective treatments for shrimp infected with *Fusarium* have been developed. Malachite green oxalate at concentrations of 0.05 to 0.1 ppm for 24 hours appeared to be effective against exposed spores and hyphae that were present in the water and tanks, but internal hyphae and spores were not affected by this treatment. Control of the disease in the raceway system described was accomplished by elimination of sources of spores of the fungus and by destruction of shrimp infected with the fungus.

Bacterial Infections

Vibrio infections have been implicated as a major cause of mortality in juvenile penaeids in shrimp culture (Sindermann, 1971; 1974). *Vibrio parahaemolyticus*, the cause of an infectious food poisoning syndrome in Japan (Nickelson and Vanderzant, 1971), was isolated from white shrimp (*P. setiferus*) taken from Galveston Bay, Texas (Vanderzant, et al., 1970a). The same organism was pathogenic to brown shrimp (*P. aztecus*) when bits of frozen white shrimp infected with the organism were fed to brown shrimp, or when cultures of the organism were added to aquaria with brown shrimp.

Lewis (1973a) reported experiments in which adult brown shrimp were challenged with a field isolate of *Vibrio anguillarum*. One-tenth milliliter of a 24-hour broth culture diluted 100-fold and introduced by injection beneath the dorsal carapace at the terminus of the rostral groove caused death of the shrimp within 5 days.

The normal microbial flora of brown and white shrimp from the Gulf of Mexico and from pond-reared brown shrimp has been studied (Vanderzant, et al., 1970b; Vanderzant et al., 1971). In these studies *Vibrio* spp. were among the predominant isolates from pond-reared brown shrimp, but were apparently not a significant part of the normal flora of brown and white shrimp from the Gulf of Mexico.

Vibrio alginolyticus has been implicated as the cause of several large mortalities during 1972 and 1973 in hatchery-reared brown and white shrimp at the Dow Chemical Company shrimp hatchery in Freeport and at the National Marine Fisheries Service laboratory in Galveston, Texas. In the most severe epizootic, a 99% loss occurred over a 2-week period in a group of 100,000, 26mm (total length) brown shrimp (Lightner and Lewis, in press). *V. alginolyticus* was also frequently isolated from dead or dying wild brown, white and pink shrimp obtained from Galveston area commercial bait dealers. Other opportunistic bacterial species such as *V. algosus*, *V. Anguillarum*, *Aeromonas* sp., and *Pseudomonas* spp. have occasionally been isolated from dead or dying hatchery-reared or wild shrimp, but *V. alginolyticus* was

Table 1. Organisms isolated and source of penaeid shrimp exhibiting clinical signs of a bacterial septicema.

Organism	Species of shrimp[1] and total length	Source, number of shrimp affected,[2] and percent mortality
Vibrio alginolyticus	B (40mm)	Lab-reared (NMFS); 150; 20%
V. alginolyticus, V, anguillarum	W (100mm)	Live bait dealer (Galv.); 50; 50%
V. alginolyticus	B (26mm)	Lab-reared (Dow); 100,000; 99%
V. alginolyticus	B (58mm)	Lab-reared (NMFS); 200; 40%
V. anguillarum, Aeromonas sp.	B (90mm)	Lab-reared (NMFS); 10; 10%
V. alginolyticus	W, P, B (100mm)	Live bait dealer (Galv.); 60; 50%
V. alginolyticus	B (41mm)	Lab-reared (NMFS); 1,500; 99%
Vibrio sp.	W, P (120mm)	Live bait dealer (Galv.); 100; 10%
V. alginolyticus, Beneckea sp.*	B (10mm)	Lab-reared (Dow); 1,035,000; 64%
V. algosus	W (100mm)	Live bait dealer (Galv.); 14; 100%
V. alginolyticus, Pseudomonas sp.	B (5mm)	Lab-reared (NMFS); 500,000; 35%
V. anguillarum, A. formicans	B (80mm)	Lab-reared (NMFS); 3; 100%

[1] B=brown shrimp (*Penaeus aztccus*), W=white shrimp (*P. setiferus*), and P=pink shrimp (*P. duorararum*). Average total length in parentheses.
[2] NMFS=National Marine Fisheries Service, Galveston, Texas, Dow=Dow Chemical Co. experimental shrimp hatchery and rearing unit, Freeport, Texas, and Galv.=Galveston, Texas.
* Possesses chitinase activity.

the most prevalent organism isolated from shrimp that showed clinical signs of a bacteremia (Table 1).

All the the bacteria isolated from the hemolymph of moribund shrimp were Kovac's oxidase positive, were motile by polar flagella, and initially required the presence of at least 2% NaCl in the medium for growth. None of the fermentative bacteria produced gas in glucose, lactose, sucrose, or mannitol. Those organisms which failed to produce lysine decarboxylase were beta hemolytic on 5% bovine blood agar and on the basis of their ability to produce arginine dihydrolase, 2–3 butanediol, gelatinase, and indole were identified as *Aeronomas* sp. (Eddy, 1969; Eddy and Carpenter, 1964; Schubert, 1967). Those organisms identified as *Vibrio* sp. were sensitive to 2, 4-diamino-6, 7-diisopropyl pteridine phosphate (Schubert, 1962); produced lysine, ornithine decarboxylase, and indole; fermented sucrose; and grew in trypticase soy broth containing 10% NaCl. Further identification of the isolates was accomplished using methods described by Lewis (1973b).

The first apparent clinical sign of a lethal bactermia was a gradual change from the usual colorless translucent appearance of the musculature, to a whitish-opaque coloration. Some infected animals examined also showed melanized cuticular erosions, and melanization of gill filaments and ventrolateral edges of the carapace. A slight darkening of the dorsal portions of the integument (due to expansion of integumental melanophores) and a reddening of the pereiopods and the pleopods (due to expansion of integumental erythrophores) was apparent in moribund or freshly dead shrimp with a bacteremia. Moribund shrimp commonly exhibited a pronounced dorsal flexure of the abdomen with the second and third abdominal segements at the apex of the flexure.

Behavioral signs of stress associated with the disease became more apparent as the disease progressed. These signs included reduced swimming activity, disorientation while swimming, and swimming on one side. Eventually, affected shrimp came to rest motionless on the bottom, some in an upright position supported by the pereiopods, pleopods, and uropods, while others lay on their side. Some of these shrimp could be induced to brief periods of swimming activity by prodding. Death usually occurred 2 to 4 hours after the shrimp had become lethargic. Occasionally shrimp remained in the upright position even after death.

Hemolymph drawn with a tuberculine syringe directly from the heart of moribund shrimp having a bacteremia was slightly turbid in appearance and lacked the blue coloration that appears in clotted hemolymph of healthy shrimp. Typically, the hemolymph from moribund shrimp having a bacteremia required more time to clot than the hemolymph from healthy shrimp, and often it did not clot at all. Giemsa-stained hemolymph smears from moribund bacteremic shrimp contained hemocytes although in greatly reduced numbers compared to normal shrimp. Gram-stained hemolymph smears from the same animals contained numerous Gram-negative rods. Pure cultures of bacteria could usually be obtained from hemolymph drawn directly from the heart of

bacteremic moribund shrimp. Cultures made from impression smears of small pieces of muscle tissue aseptically removed from the abdomen of small shrimp (under 40mm total length) also frequently provided pure cultures of the presumed causative agent. Isolation medium was tryptic soy agar with 2% NaCl.

Addition of bacterial isolates to aquarium water or feeding of bacterial isolates to experimental shirimp seldom resulted in clinical disease. Clinical disease could only be produced by direct injection of about 10^4 bacterial cells into the abdominal muscle or hemocoel of an experimental shrimp. Other investigators have reported similar difficulties in infectivity experiments with decapod crustaceans. Barkate (1972) was unable to infect juvenile pink shrimp with *V. parahaemolyticus* when added to tank water at 10^4 cells per milliliter. Lewis (1973a) for the same reason selected injection of *Vibrio anguillarum* into experimental shrimp over other methods of exposure. Sniesko and Taylor (1947) were unable to infect American lobsters with *Pediococcus (Gaffkya) homari* introduced with the food, but succeeded in transmitting geffkemia disease to healthy lobsters by injection of bacteria. Later it was learned that the gaffkemia organism is transmitted only through ruptures in the integument and not through the consumption of infected food (Stewart and Rabin, 1970).

Our experience has shown that handling of otherwise healthy hatchery-reared shrimp occasionally results in the onset of a bacteremia due in most cases to a *Vibrio* sp. In all cases some sort of physical or chemical stress or injury preceded the onset of clinical disease. The capture and holding in tanks of wild penaeid shrimp often result in the same disease syndrome. Slight injuries resulting in interruption of the cuticle certainly occur when shrimp are subjected to rough handling or crowding in tanks. Cuticular injuries may provide a route of entry for potentially pathogenic bacteria which are a normal part of the microbial flora of pond-reared or hatchery-reared shrimp (Vanderzant, et al., 1970b; Vanderzant, et al. 1971).

Treatment of *Vibrio* infections is possible the addition of antibiotics to the ration or directly to the water. Experimental groups of brown shirimp fed Terramycin[1] at the rate of 360–387 mg/kg body weight/day for 14 days suffered less mortality than comparable control groups not fed antibiotic when challenged by direct intramuscular inoculation of at least 10^4 cells of *Vibrio alginolyticus* per shrimp (Corliss, et al., in press). Chan and Lawrence (in press) reported the effectiveness of oxytetracycline-oleandomycin combinations in reducing bacterial populations in larval shrimp cultures and suggested that the antibiotic combination could be used to treat *Vibrio* and other bacterial infections in mysis and postlarval stage shrimp. Delves-Broughton (1974) reported that the broad spectrum anitibiotic Furanace when added directly to the water is non-toxic and is rapidly absorbed into the tissue to treatment levels in *Macrobrachium rosenbergii.* The *Vibrio* spp. tested were all inhibited "in vitro" by less than 1 mg Furance/liter. *Aeromonas* and *Cytophaga* spp. were inhibited by slightly higher concentrations of the chemotherapeutic (0.8 to 3.1 mg/liter). *Beneckea* spp. showed a varied response (3.1 to 12.5 mg/liter) while *Pediococcus (Gaffkya) homari* were resistant.

Shell Disease

Chitinoclastic bacteria are apparently a normal part of the microbial flora of the penaeids (Hood and Meyers, in press). However, several species of *Beneckea, Vibrio,* and *Pseudomonas* that produce chitinase have been isolated from shrimp exhibiting "shell disease" (Cook and Lofton, 1973). "Shell disease" was described by Rosen (1970) as a complex of closely related low virulence necrotic diseases of the integument of aquatic crustaceans. Rosen (1970) noted that chitinoclastic bacteria and fungi have been implicated as causative agents of the disease.

In 1973 an epizootic of shell disease developed at the experimental shrimp farm at Puerto Peñasco, Mexico. The epizootic appeared to be caused by a chitinoclastic variety of *Vibrio anguillarum.* In contrast to the forms of shell disease described by Cook and Lofton (1973) and Rosen (1970), the Peñasco variety of shell disease was accompanied by low but persistent daily mortalities of 1 to 5%. The disease appeared to be infections and not the result of secondary infections of wounds.

The lesions seen in the Puerto Peñasco varietly of shell disease occurred consistently in certain locations, namely on the dorsal surface of the pleura of the first, second, and third abdominal segments, on the posterior edge of the lateral portions of the abdominal pleural plates, and along the dorsal surface of the branchial cavity (Figs. 10 and 11).

Shell disease at Puerto Peñasco has been treated experimentally with 1-hour static treatments of Hyamine 3500 at 5 ppm, potassium permanganate at 10 ppm, and mixtures of malachite green oxalate and formalin at 0.05 to 0.1 ppm and 20 to 75 ppm, respectively. Mixtures of malachite green oxalate and formalin at these levels have been effective in preliminary experiments in reducing losses due to shell

[1] Use of trade names in this publication does not imply endorsement of commercial products.

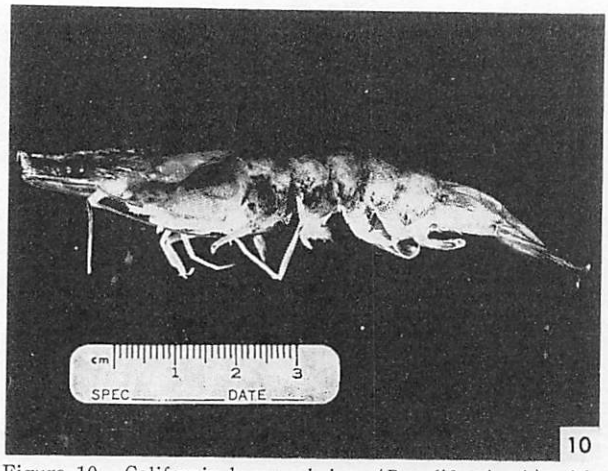

Figure 10. California brown shrimp (*P. californiensis*) with the Peñasco variety of shell disease. Melanized cuticular lesions typical of this from of shell disease are located on the plates of the abdomen, and in the dorsal portion of the branchial chamber.

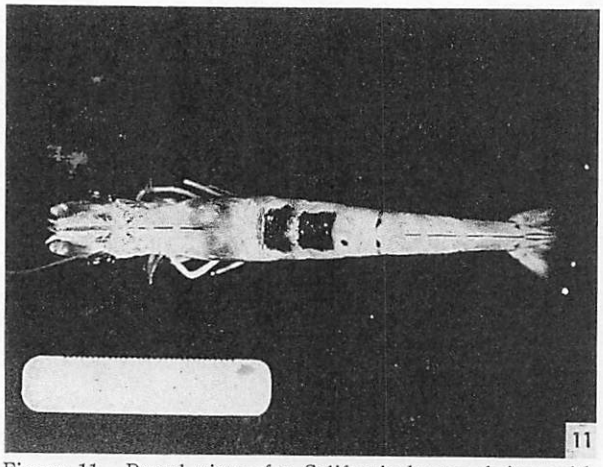

Figure 11. Dorsal view of a California brown shrimp with shell disease. The lesions shown on the dorsal portions of the first row abdominal segments were common in Peñasco shell disease epizootics.

disease. Following this treatment, shrimp having the disease molted within a few days and the new cuticle was free of lesions. Hyamine 3500 and potassium permanganate were not effective against shell disease at the concentrations tested.

Terramycin when added directly to the ration (20g Terramycin per 45kg ration; ration fed at approximately 10% of the biomass per day for 14 days) was also effective in a single preliminary experiment in treating shrimp having shell disease. Further studies using mixtures of malachite greeen oxalate-formalin and Terramycin (separately and together) as treatments for this form of shell disease are needed.

Gill Disease

Gill disease in penaeid shrimp is a complex of several diseases, any of which may result in death of affected shrimp by destruction of the gills or by suffocation resulting from mechanical blockage of gas exchange across the surface of the gill lamellae. Organisms demonstrated to cause gill disease in penaeids include species of imperfect fungi belonging to the genus *Fusarium*, at least two types of ectocommensal peritrichs that belong to the genera *Zoothamnium* and *Lagenophrys*, and a filamentous bacterium that superficially resembles *Laucothrix mucor*.

"Black gills" or "black gill disease" has been described from several decapod crustaceans besides the penaeids (Johnson, 1974a; Egusa and Ueda, 1972; Uzman and Haynes, 1968). "Black gills" or melanization of the gill processes is a clinical sign of some types of gill disease, but is not a disease in itself. "Black gills" occur in shrimp having fungus infections of the gills (Egusa and Ueda, 1972; Uzman and Haynes, 1968), and in shrimp with heavy infestations of *Lagenophrys*.

"Black gills" are not usually seen in animals that have heavy infestations of the ectocommeasal peritrich *Zoothamnium* or of the filamentous bacterium on the surface of the gills. Detritus and algae are often trapped by these ectocommensals resulting in gills that range in color from green to dark brown, but usually the gills are a very pale brown or colorless.

Gill Disease Due to Fusarium sp.—Gill disease due to species of *Fusarium* has been discussed earlier and is often accompanied by black gills.

Gill Disease Due to Epicommensal Protozoans.—Johnson et al. (1973) reported the loss of an estimated 2,000 pond-held brown and white shrimp in a single day due to the presence of large numbers of *Zoothamnium* sp. (Fig. 12) on the gills and to a reduction in dissolved oxygen. Mortality was attributed to anoxia as the mortalities occurred when the infestation of the protozoan became heavy enough to restrict oxygen exchange, and when the dissolved oxygen level in the ponds dropped below 3 ppm to a low of 2.6 ppm. In ponds where no *Zoothamnium* sp. were observed on the shrimp, no mortalities occurred despite the low dissolved oxygen levels. A dissolved oxygen level of 2.6 ppm is not normally lethal. Good survival has been experienced with *P. aztecus* in culture ponds even when the dissolved oxygen fell to 1 ppm.

Histopathological lesions of the gills, appendages, or of the general body surface have not been demonstrated at the site of attachment of a colony of *Zoothamnium* sp.. The stalks of colonies of this protozoan attach to the surface of the cuticle and do no mechanical damage. There is no foreign body response at the site of attachment by the shrimp's hemocytes (Fig. 13). Death occurs when the effective respiratory surface of the gills is reduced by the presence of

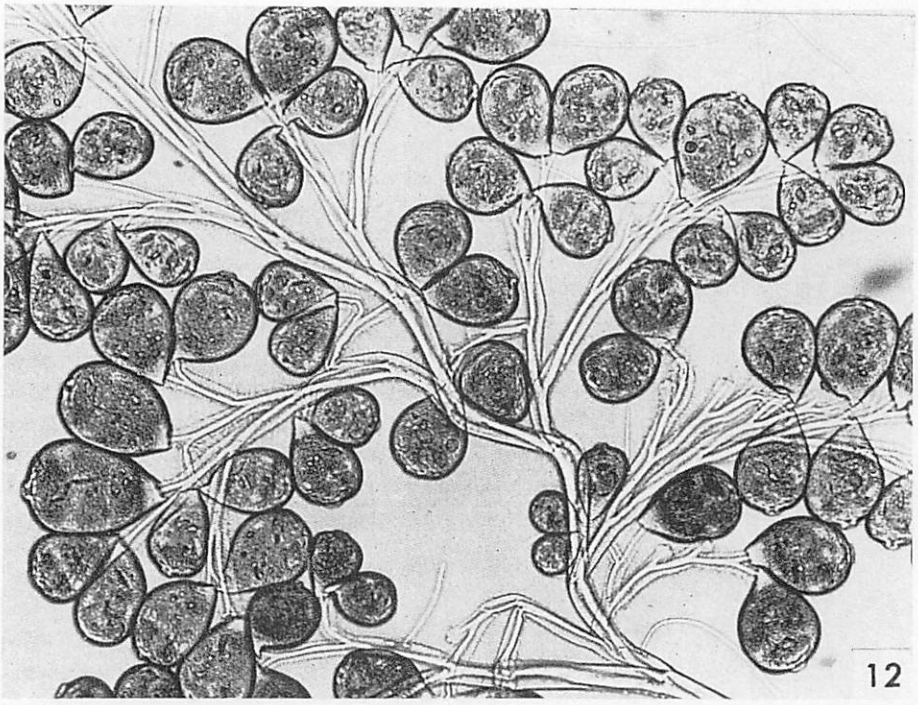

Figure 12. Photomicrograph of a wet mount preparation of *Zoothamnium* sp. from the gills of a brown shrimp (*P. aztecus*). No stain. ×320.

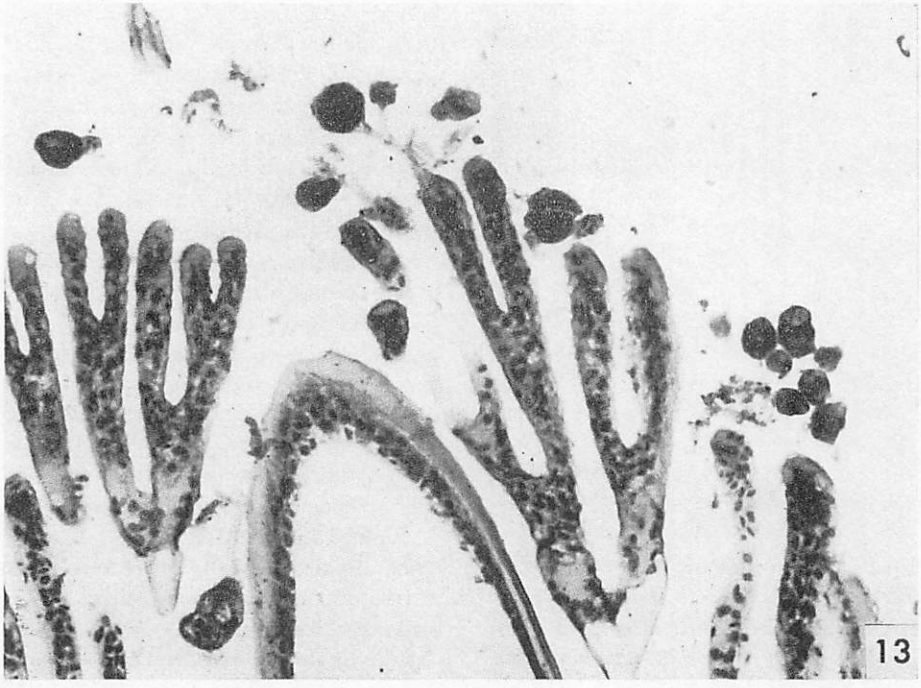

Figure 13. Histological section of the gills of a white shrimp (*P. setiferus*). Colonies of *Zoothamnium* sp. are attached to the cuticle of the gill lamellae but host response is absent. Hematoxylin and eosin. ×250.

mumerous colonies of *Zoothamnium* sp. and suffocation results. The process is passive and is probably aggravated by reduced dissolved oxygen concentrations in the water (Overstreet, 1973).

Successful control of *Zoothamnium* sp. on penaeid shrimp in ponds with formalin at 25ppm was reported by Johnson et al. (1973). A lower concentration of formalin (15 ppm), potassium permanganate at 2 and 4 ppm, ccpper sulfate at 1 ppm, and malachite green at 1 ppm were not effective in other experiments in killing or removing *Zoothamnium* colonies from the shrimp's gills.

A loricate peritrich, probably a *Lagenophrys* sp., has been observed on a general body surface of pond-reared shrimp (*P. setiferus* and *P. vannamei*) in Texas (Johnson, 1974a). At Galveston a similar *Lagenophrys* sp. was observed on the gills of white and brown shrimp. When present on the gills, *Lagenophrys* sp. differs from *Zoothamnium* sp. by evoking a strong cellular inflammatory response. Individual trophonts of *Lagenophrys* sp. typically attach near the tips of the gill lamellae (Fig. 14). While no portion of the lorica appears to penetrate or damage either the cuticle or the underlying hypodermis of the lamellus, the site of attachment becomes heavily inflamed and congested with hemocytes (Fig. 15). Often the hemocyte accumulations become melanized. A similar process of inflammation by hemocytes was noted in the processes of wound repair and foreign body elimination in the white shrimp (Fontaine and Lightner, 1973; 1974). Shrimp having heavy infestations of *Lagenophrys* sp. on the gills display a "black gill" condition. In such animals, numerous gill lamellae and often large portions of a whole gill process are heavily congested with hemocytes, melanized, and are nonfunctional. Hence, the respiratory capacity is reduced, and in severely effected animals, death due to suffocation may result if tissue oxygen demands increase (e. g., following handling stress or immediately prior to molting) or if dissolved oxygen levels decrease.

Filamentous Gill Disease.—Leucothrix mucor and *Leucothrix*-like filamentous bacoeria have been reported from numerous crustaceans. The presence of *Leucothrix mucor* has been demostrated on the eggs of the rock crab (*Cancer irroratus*), on the setae of the pleopods of the grass shrimp (*Palaemonetes pugio*) and the green crab (*Carcinus maenas*) (Johnson et al., 1971). *Leucothrix*-like filaments have been reported on the surface of developing prawn (*Palaemon serratus*) eggs and on the setae of the pleopods (Anderson and Conroy, 1968). Johnson (1974a) reported a *Leucothrix*-like filamentous bacterium on the general body surface and on the gills of three penaeid species (*P. stylirostris, P. setiferus*, and *P. vannamei*) from rearing ponds in Texas. Occasional heavy infestations of this bacterium were noted on the gills.

Barkate et al. (in press) reported mortalities in postlarval penaeid shrimp due to a large filamentous

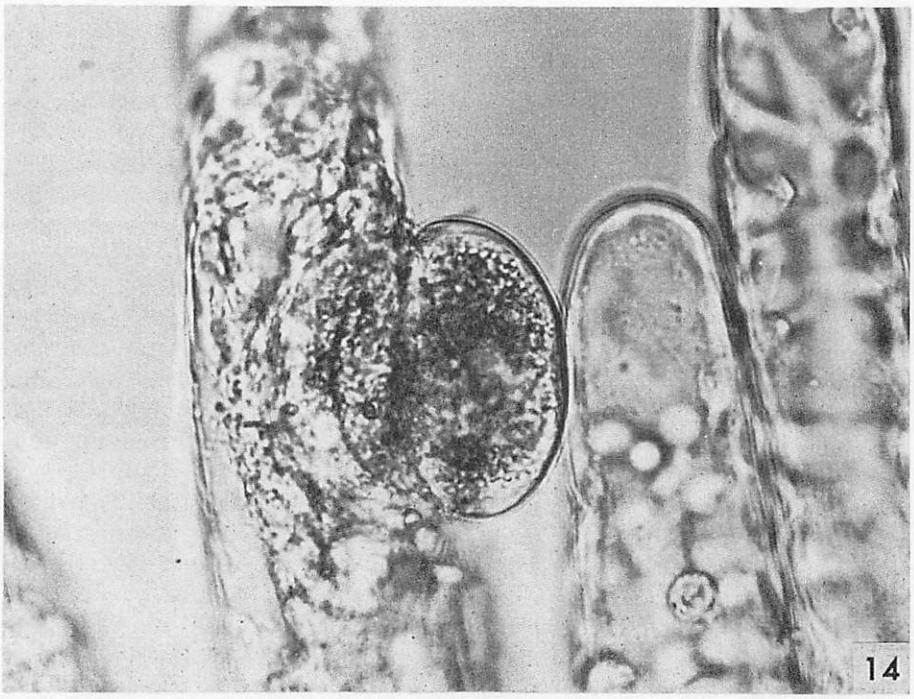

Figure 14. A *Lagenophrys* sp. attached to the cuticle near the distal end of a gill lamella of *P. aztecus*. No stain. ×640.

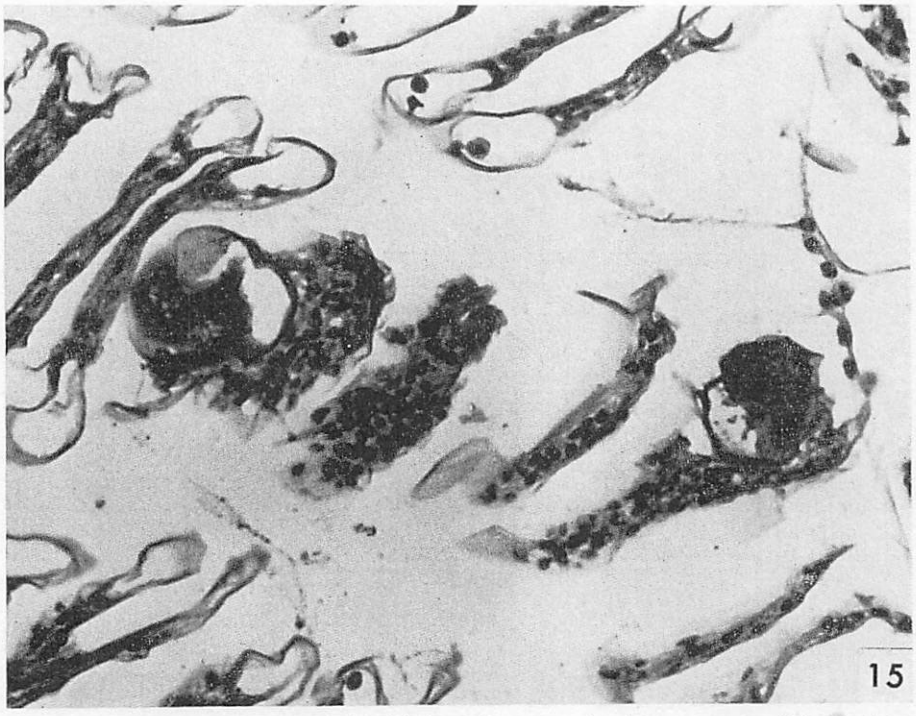

Figure 15. Histological section of the gills from *P. aztecus* showing a strong cellular inflammatory response to the two trophonts of *Lagenophrys* sp. shown. Hematoxylin and eosin. ×400.

bacterium. During early stages of development, postlarval shrimp became entangled in filaments of the bacterium, and this entanglement resulted in stress to the shrimp. Direct attachment of the filaments to the carapace region of the postlarval shrimp was also observed. Heavy mortalities (30 to 100%) were experienced, usually suddenly and without warning except for a foul sewage-like odor to the tank. This filamentous bacterium apparently grew on waste materials on the bottom of tanks and was visible as white, cottony mats on the surface of the sediment. The organism was successfully isolated and cultured, but failed to produce filaments on culture media. Reversion to the filamentous state was reportedly not accomplished unless the cultures were inoculated back into the shrimp environment (Barkate et al., in press). In culture Barkate's isolate produced spore-forming rods that stained Gram-positive to Gram-variable. The isolate was tentatively identified as *Bacillus cereus* var. *mycoides*. Infection of healthy postlarval brown and white shirimp was accomplished by addition of the isolate to beakers containing 800 ml of sterilized seawater and 15 shrimp. All of the shrimp which were exposed to the filamentous bacterium died within 48 hours, whereas the control shrimp survived for the duration of the experiment (7 days). The bacterium covered much of the surface of the shrimp at death (Barkate, et al., in press).

A similar filamentous organism was observed on the gills of juvenile penaeid shrimp in rearing tanks in Florida, and this organism was reportedly isolated on solid media. However, attempts to infect healthy shrimp using "infested tank sediment" rather than the isolate rom the gills were not successful (Barkate et al., in press).

Sporadic but serious epizootics due to a *Leucothrix*-like filamentous organism have occurred in tank- and raceway-reared *P. californiensis* at the experimetal shrimp farm in Puerto Peñasco, Mexico. Periodic samplng of affected populations of shrimp from the tanks and raceways at Peñasco revealed that the filamentous organism is typically present on the pleopods and gills. When the filamentous organism became so abundant on the gills that respiration was blocked, mortality occurred (Fig. 16).

The filamentous organism at the Puerto Peñasco shrimp farm was initially thought to be either a species of the Oscillatoriaceae group of the blue-green algae or a filamentous bacterium. The latter is believed to be the case, particularly because of the close morphological similarities of this organism to Barkate's isolate (Barkate et al., in press), *Thiothrix marina* (Harold and Stanier, 1955), and *Leucothrix mucor* (Johnson et al., 1971; Bland and Brock, 1973).

The possibility that the filamentous organism in

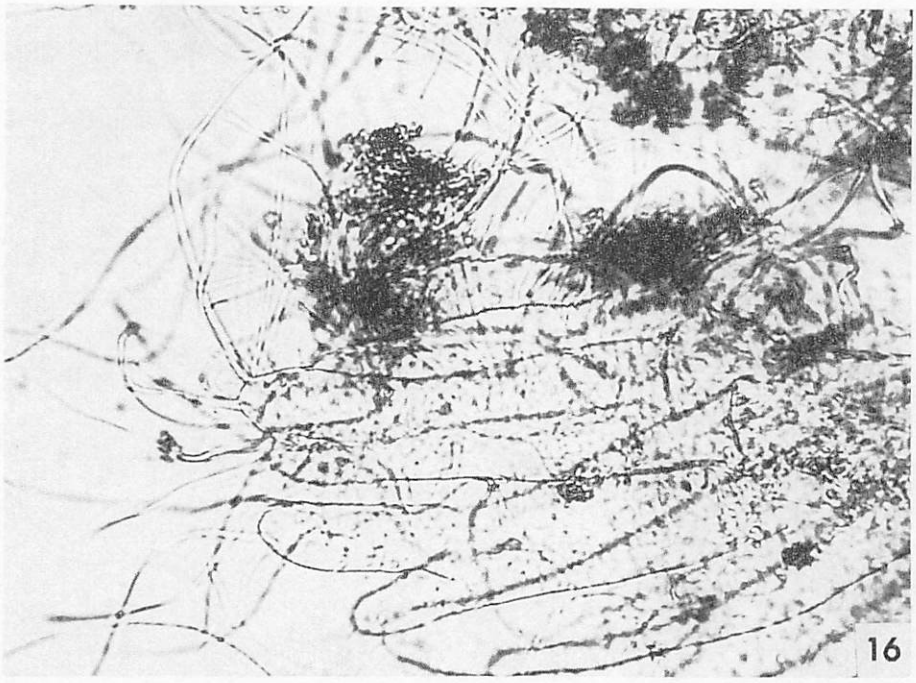

Figure 16. Wet mount preparation of the gills of a brown shimp (*P. aztecus*) heavily infested with a *Leucothrix*-like filamentous bacterium. Dark areas are debris. No stain. ×400.

Puerto Peñasco may be a blue-green alga was considered because Shelton (1974) described a blue-green alga on the chemoreceptor setae of the North Atlantic brown shrimp (*Crangon crangon*). This organism was similar in morphology to the filamentous organism on the California brown shrimp (*P. californiensis*) in Puerto Peñasco. Shelton (1974) did not culture or attempt to classify the alga from *C. crangon*, beyond placing it in the Oscillatoriaceae. However, a blue-green alga has not been obtained in culture from shrimp having filamentous gill disease.

Histological studies performed on California brown shrimp (*P. californiensis*) from the Puerto Peñasco facility and on brown and white shrimp from Galveston, Texas, revealed that the filamentous organism was strictly external. The organism was attached to the cuticular covering of the gill lamellae, pleopods, or other appandages, and its presence did not result in demonstrable histological damage to underlying tissues (Fig. 17). The filaments themselves appeared to be segmented and had the general appearance of a string of tubular beads (Fig. 18), particularly in histological preparations. The filament segments were anucleate. Trapped in the "mat" formed by the filaments were abundant amounts of detritus, some filamentous green algae, and often numerous diatoms. By itself the filamentous organism is not responsible for discoloration of the gills, but accumulation of algae and debris trapped by the filaments results in discoloration of the gills that ranges from pale brown to black or green if sufficient algae is trapped by the filaments.

The conditions responsible for the presence of the filamentous organism on the shrimp's gills have not been determined, although low dissolved oxygen levels seem to favor development of the disease, as well as contributing to mortality once the disease has become established. Mortality of shrimp having heavy infestations of the filamentous ornanism on the gills usually occurs during or immediately following molting. Most of the daily mortalities occurring in epizootics of filamentous gill disease at Puerto Peñsco were "soft shelled". Many premolt animals that showed heavy gill infestations did not survive the next molt if left untreated, despite apparently adequate, but less than saturated dissolved oxygen levels in the water. Animals that did survive the molt shed the filaments with the cast exoskeleton and remained free of the filaments for at least a few days.

Attempts to culture the organism of filamentous gill disease have been made using mumerous media, some that are intended for culture of blue-green algae, some for fungi, and the remainder for various types of bacteria. Unfortunately, on organism has been cultured that produces filaments when grown on artificial media that are like those seen in filamentous gill disease. However, one particular

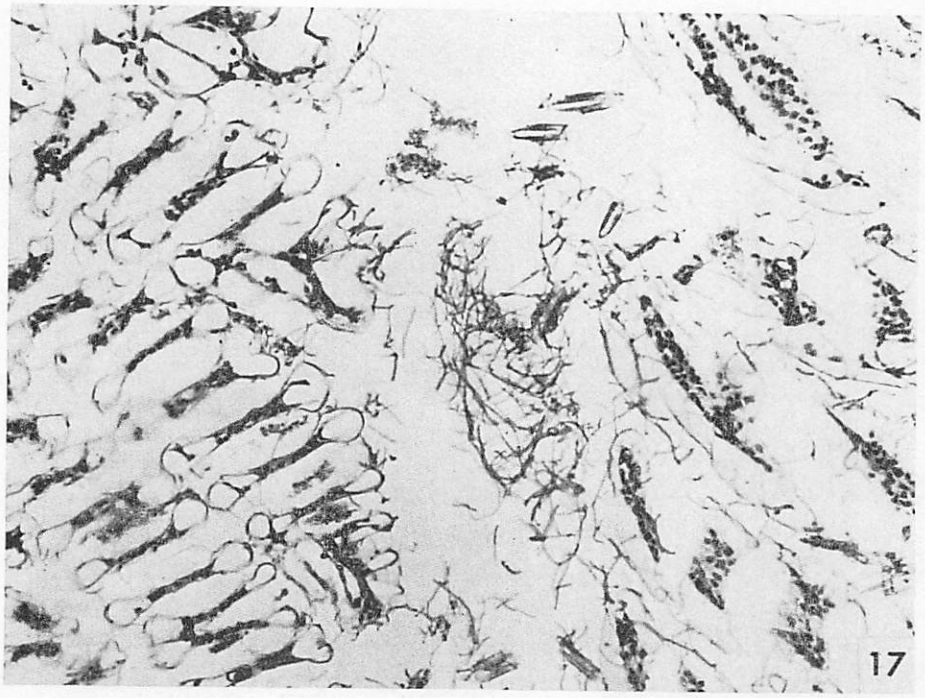

Figure 17. Histological section of the gills of a brown shrimp (*P. aztecus*). Filaments of the *Leucothrix*-like bacterium are abundant on and between the lamellae which show no inflammatory response or histo-pathological change. Hematoxylin and eosin. ×180.

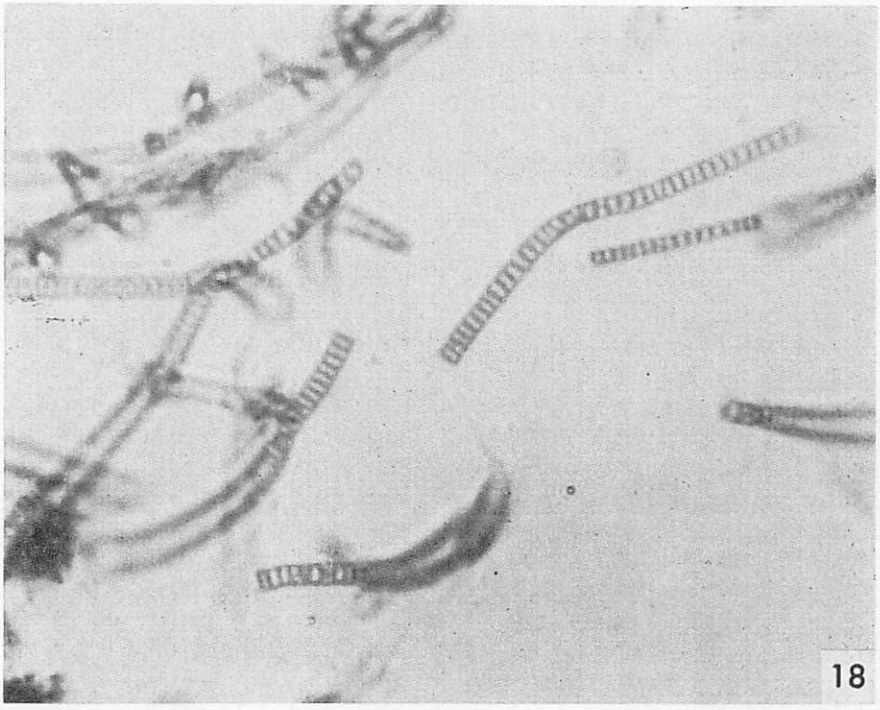

Figure 18. Highly magnified photomicrograph of the *Leucothrix*-like filamentous bacterium from the gills of a California brown shrimp (*P. californiensis*). Note the "string of beads" appearance of the filaments. Formalin fixation, hematoxylin and eosin. ×2,000.

organism has been consistently present in cultures obtained from shrimp having filamentous gill disease in Galveston and in Puerto Peñasco. This orgnism attracted our attention because it liquified agar, and hence its colonies, when present on certain agar media, lie in pits, while the other organism present do not.

The results of some preliminary experiments indicate that the organism that forms pits on certain agar media such as Harold and Stanier's medium (Harold and Stanier, 1955) is at least associated with filamentous gill disease. When cultured, this organism does not closely resemble the filamentous gill disease organism although it does form long chains and filaments. Additional work is needed to determine whether or not this organism is the causative agent of filamentous gill disease. This organism may belong to the genus *Cytophaga*, to which several pathogens of fish also belong. One disease of hatchery-reared salmonids apparently caused by *Cytophaga* sp. is bacterial gill disease (Bullock, 1972).

Shrimp having filamentous gill disease have been treated successfully with 5 to 10 ppm potassium permanganate in 1-hour static treatments. Occasionally, the use of 10 ppm potassium permanganate has resulted in a chemical toxicity expressed as "burned" gill lamellae which became melanized, necrotic, and were later sloughed. A concentration of 5 ppm potassium permangante removed or greatly reduced the filaments on the gills, but unfortunately within 5 to 10 days filaments reappeared and mortalities began again. Other chemical treatments including mixtures of malachite green oxlate and formalin at 0.005 to 0.1 ppm and 25 to 75 ppm, respectively, and Hyamine 35000 at 1 to 2 ppm have not been beneficial an 1-hour static treatments. Chemicals such as methylene blue, Roccal, copper sulfate, and Furanace are presently being tested. Also being tested for effectiveness against filamentous gill disease are 24-hour flow-through treatments using 1 to 2 ppm potassium permanganate.

Microsporidian Diseases

The microsporidian parasites of shrimp pose a serious threat to shrimp in open-culture systems such as ponds or the intertidal zones of bays. Aquaculture ventures using tanks or raceways, particularly those operating as closed or semiclosed systems, have not been affected by microsporidian parasites.

At least four species of microsporidia are parasitic to the penaeids of North America and all four have been described from only the Gulf of Mexico and South Atlantic states. *Nosema nelsoni* commonly infects brown and white shrimp (Overstreet, 1973) and the pink shrimp in Florida (Hutton et al., 1959). Because *N. nelsoni* occurs more commony than other microsporidia in wild penaeids in the Gulf of Mexico, it may become a more serious problem in commercial shrimp culture operations than will other species of the microsporidia. Shrimp infected with *N. nelsoni* are known as "milk" or "cotton shrimp" due to the chalky white appearance and the "cottony" texture of the musculature (Fig. 19). Histologically, the

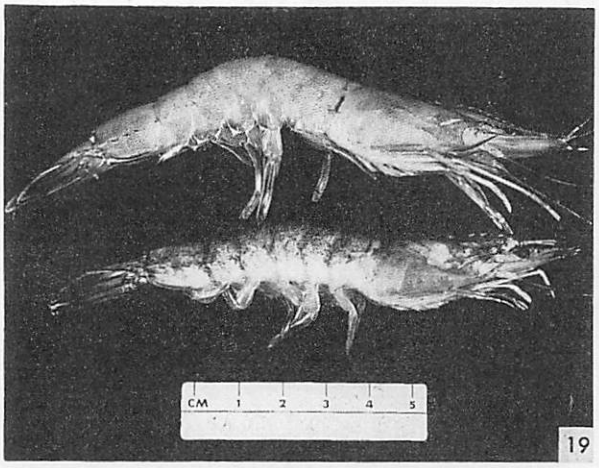

Figure 19. Comparison of a normal shrimp (top) with a "cotton shrimp" (botton). The cotton shrimp is infected with the microsporidian, *Nosema nelsoni*.

striated muscle fibers become nearly surrounded and eventually are replaced by masses of spores of the parasite (Fig. 20). The fresh spores of *N. nelsoni* are single and average 2.5 μm in length by 1.5 mm in width (Overstreet, 1973). Cotton shrimp disease is chronic and gradually debilitates the host. Severely affected animals are common in infected populations of shrimp, but these individuals are more apt to be taken by predators than normal shrimp and are much less resistant to stress. Such shrimp typically do not survive handling, while uninfected shrimp from the same population show good survival. From a commercial viewpoint, cotton shrimp are not a desirable product.

An incidence of 16% cotton shrimp was reported by Elam (personal communication, Texas Parks and Wildlife Department, Palacios, Texas) in 120–130 mm (total length pond-reared brown shrimp in June of 1972. Marifarms in Panama City, Florida, experienced an incidence of about 15% cotton shrimp at harvest in 1971 in white shrimp reared in a net enclosed bay (J. Ikeguchi, personal communication, Marifarms, Panama City, Florida). Since 1971, the incidence of cotton shrimp at Marifarms has been less than 1%.

Another microsporidian, *Pleistophora* sp., causes a similar form of cotton shrimp disease that is grossly

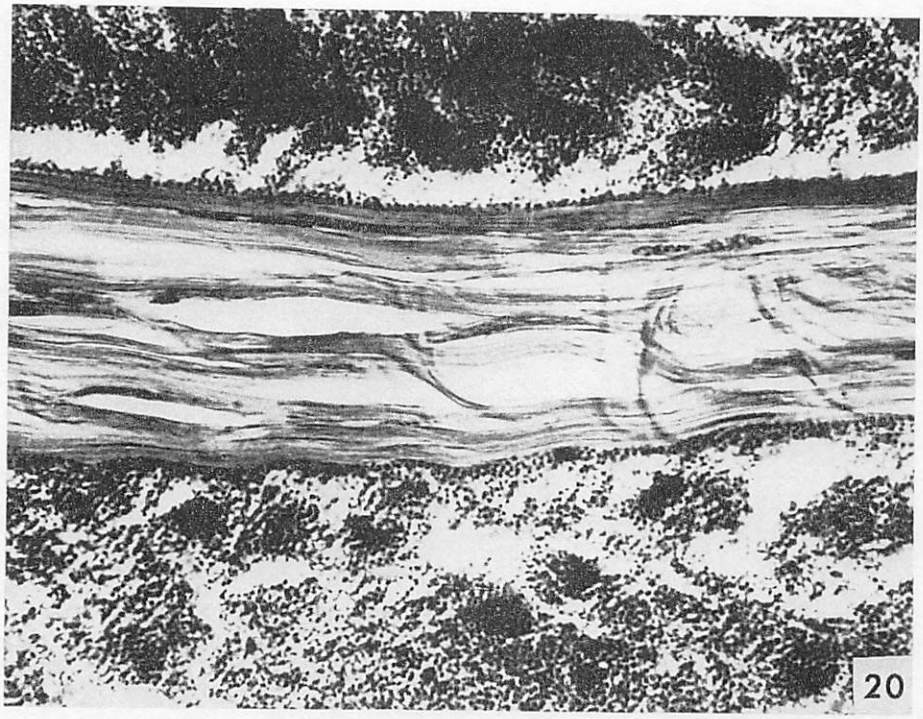

Figure 20. Histological section of tail muscle from a shrimp infected with *Nosema nelsoni*. Giemsa's stain. ×900 (approximate).

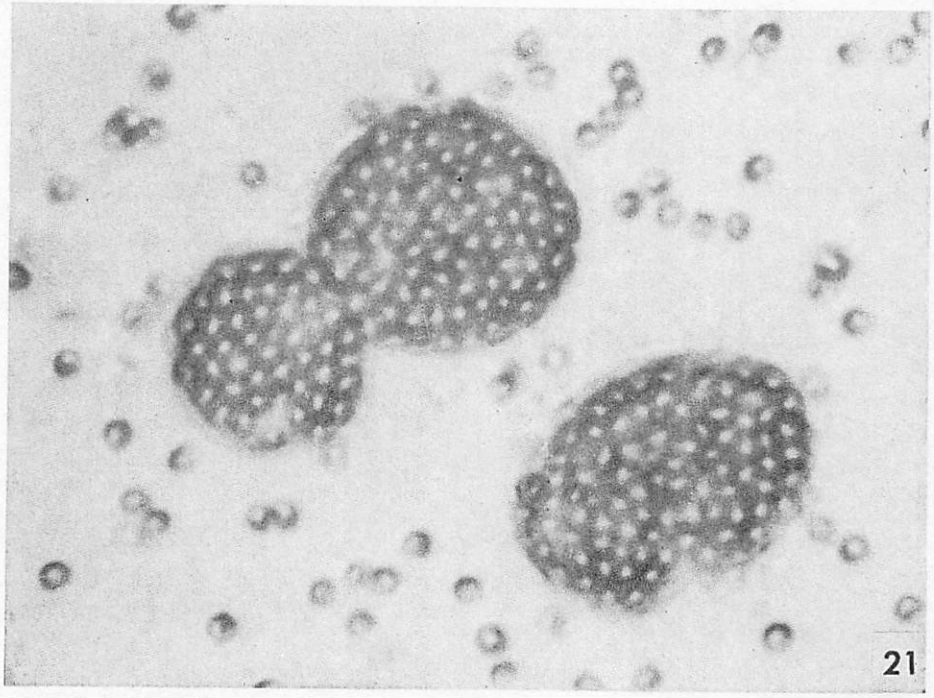

Figure 21. Impression smear of *Pleistophora* sp. from the brown shrimp (*P. aztecus*). Giemsa's stain. ×1300 (approximate).

indistinguishable from infections caused by *N. nelsoni* (Baxter et al., 1970; Overstreet, 1973). The disease has been reported in the brown and white shrimp from Texas (Baxter et al., 1970). Spores of *Pleistophora* sp. develop in sporonts or cysts that are 10 to 55 μm in diameter and contain 14 to hundreds of spores (Fig. 21). Individual fresh spores are slightly pyriform, 2.3 to 3.0 μm long by 1.7 to 2.5 μm wide with a uniform capsule 0.5 μm wide and a polar filament 53–125 μm long by 0.3 μm wide (Overstreet, 1973). This parasite usually replaces the striated muscle tissue, but is also found occasionally in the cardiac muscle, hepatopancreas, gills, and stomach wall muscle (Baxter, et al., 1970).

Pleistophora sp. is not nearly so common in wild shrimp populations as in *N. nelsoni*. It was present in 6.4% of the cotton shrimp sampled from Galveston Bay in 1969 and 1970, while *N. nelsoni* was present in the remainder of the cotton shrimp (Baxter, personal communication, NMFS, Galveston, Texas). *Pleistophora* sp. has not been observed in cultured shrimp on the Gulf of Mexico.

Two additional species of the microsporidia are likely to occur in the culture of penaeid shrimp. These species are *Thelohania duorara* and *T. penaei*.

T. duorara has been reported from pink shrimp and from the Caribbean brown shrimp, *P. brasiliensis* (Iversen and Manning, 1959; Iversen and Van Meter, 1964; Overstreet 1973). Kruse (1959) reported what is probably the same species in pink, brown, and white shimp. Affected shrimp have a "cotton shrimp" appearance that is like that caused by *Nosema nelsoni* or *Pleistophora* sp. infections. Spores of *T. duorara* usually lie between muscle fibers, but may completely replace that tissue (Fig. 22). The heart, gonads, and nerve tissues may also be infected. When fresh, the pyriform-shaped spores (Fig. 23) range in size from 4.7 to 6.8 μm long by 3.0 to 4.2 μm wide (average 6.0 by 3.7 μm) with a uniformly wide polar filament 97 to 142 μm long, and eight such spores are in sporonts 8.5 to 13.6 μm in diameter (Overstreet, 1973).

T. penaei infections usually are located along the dorsal midline of the white shrimp. The parasite infects smooth muscles of the blood vessels, foregut, and particularly the germinal tissue of the gonads (Overstreet, 1973). In advanced infections, few if any viable sperm or ova are present in the gonads. The potential effect of this disease is obvious if it were to occur in breeding stock.

The distinctly pyriform spores of *T. penaei* are found in groups of eight in the sporont. There are both microspores which measure 2.5 to 4.7 μm long by 2.0 to 3.5 μm wide, averaging 4.0 by 2.3 μm, and megaspores which are 5.5 to 8.2 μm by 3.5 to 4.2 μm. The polar filaments protrude 65 to 87 μm, averaging 74 μm, with a uniformly thick proximal portion and a thin distal portion following a transitional tapering zone. Sporonts are 7 to 12 μm wide, averaging 9.3 μm

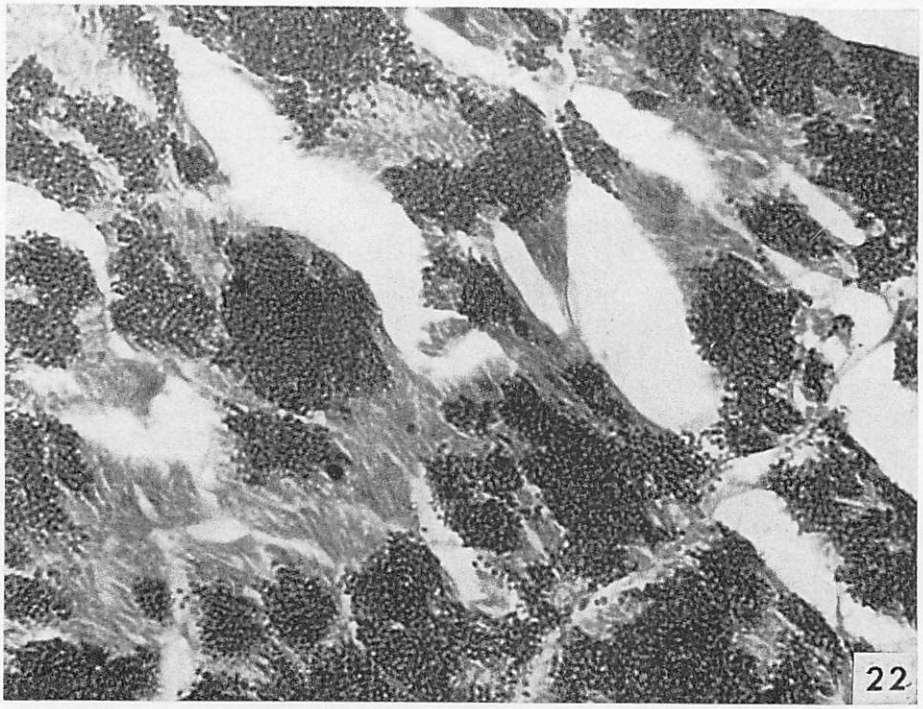

Figure 22. Photomicrograph of *Thelohania duorara* in the tail muscle of a brown shrimp, (*P. aztecus*). Giemsa's stain. ×640.

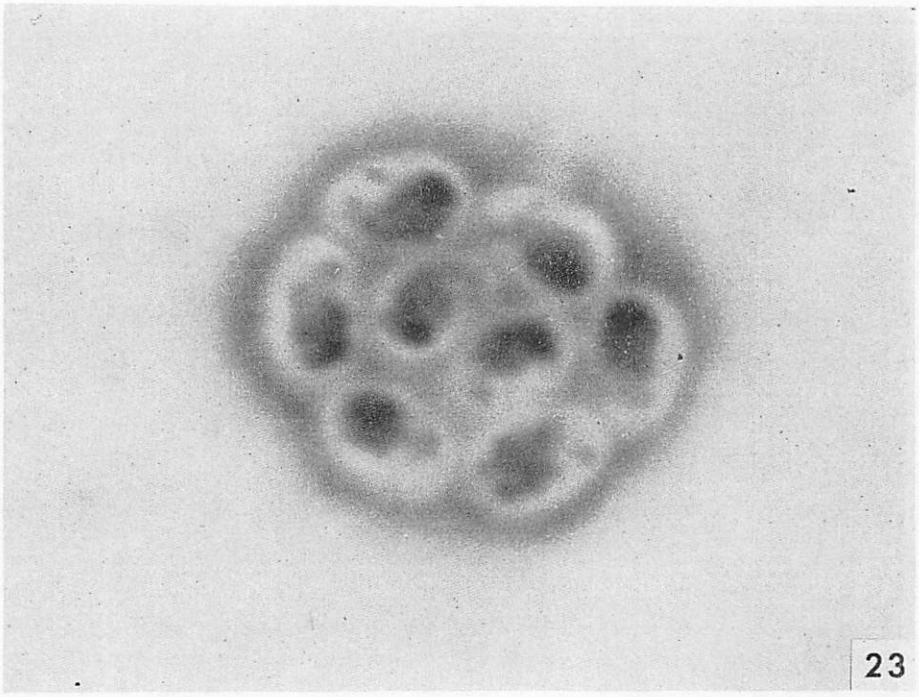

Figure 23. Photomicrograph of an impression smear of the gonad of a male white shrimp (*P. setiferus*) infected with *Thelohania penaei*. Giemsa's stain. ×5,500 (approximate).

(Overstreet, 1973).

Shrimp can be infected with more than one species of microsporidian. Individual white shrimp (*P. setiferus*) infected with *T. penaei*, *N. nelsoni*, and *Pleistophora* sp. were reported from Mississippi (Overstreet, 1973). At Galveston, brown shrimp from Galveston Bay have been found to possess dual infections of *N. nelsoni* and *T. duorura* in the tail musculature.

There are no known treatments for shrimp infected with any of the microsporidia. Furthermore, the means of transmission from host to host and even the method by which shrimp become infected remain to be discovered (Roth and Iversen, 1971).

SUMMARY

At least five major diseases of cultured penaeid shrimp are recognized as potential obstacles to successful commercial culture of penaeid shrimp. These diseases are:

1. A mycosis of larval penaeid shrimp caused by a *Lagenidium* sp. This disease has been recognized in larval white (*Penaeus setiferus*) and brown (*P. aztecus*) shrimp in Texas and in Florida. The protozoeal larval stages are the most severely affected by the fungus with losses of nearly 100% reported. No methods of chemical or drug therapy have been developed for treatment of the disease.

2. A mycotic infection of juvenile penaeid shrimp with *Fusarium* spp. have been reported from *P. japonicus* in Japan, *P. duorarum* and possibly *P. aztecus* in Texas, and in *P. californiensis* in Puerto Peñasco, Sonora, Mexico. High mortalities accompanied the disease in *P. japonicus* and *P. californiensis* and affected shrimp frequently possessed "black gill". As is the case with the mycosis caused by *Lagenidium* sp., no methods of therapy have been reported in treating shrimp infected with *Fusarium* spp., but control of the disease was accomplished at Puerto Peñasco by elimination of the source of the fungus and by destruction of infected stock.

3. Bacterial infections caused by *Vibrio* spp. and *Beneckea* spp., *V. parahaemolyticus*, *V. alginolyticus*, and *V. anguillarum* have been implicated as the cause of severe bacteremic epizootics in cultured penaeid shrimp in Texas. Losses due to *Vibrio* infections have reportedly reached nearly 100%. Certain antibiotics have possible beneficial therapeutic effects in treatment of *Vibrio* infections when added directly to the ration or to the water.

"Shell disease" is a complex of diseases expressed as cuticular lesions that typically become melanized. Chitinoclastic bacteria belonging to the genera *Beneckea*, *Vibrio*, and *Pseudomonas* have been isolated from shrimp having shell disease. As with bacteremic infections due to *Vibrio* spp., bacterial "shell disease"

has been treated successfully in preliminary expeiments in Puerto Peñasco using antibiotic therapy administered with the ration. Addition of mixtures of malachite green oxalate and formalin to the water in static 1-hour treatments has also shown a beneficial effect in preliminary tests.

4. Gill disease. Gill disease in penaeid shrimp is a complex of several diseases, all of which may result in death of affected shrimp by destruction of the gills or by sufocation due to mechanical blockage of gas exchange across the surface of the gill lamellae. Organisms demonstrated to cause gill disease in penaeids include *Fusarium* spp., *Zoothamnium* sp., *Lagenophrys* sp., and a *Leucothrix*-like filamentous bacterium. Frequent and high losses of shrimp have been experienced in Texas, Florida, and Puerto Peñasco, due to members of this complex of gill diseases. Successful therapy using formalin has been reported for pond-reared shrimp having an infestation of the gills by *Zoothamnium* sp. in Texas. The severity of epizootics of "filamentous gill" disease due to a *Leucothrix*-like filamentous bacterium in Puerto Peñasco has been temporarily reduced in raceway and tank-reared shrimp by addition potassium permanganate to the water.

5. Microsporidian disease. At least four species of microsporidia are parasitic to the penaeids of North America. *Nosema nelsoni, Pleistophora* sp., *Thelohania duorara,* and *T. penaei* cause a chronic disease in penaeids that is commonly referred to as "cotton shrimp" diasese due to the cottony appearance of the tissues infected by the parasite. No effective treatments have been reported for this group of parasites in shrimp, nor has the means of transmission from shrimp to shrimp been discovered.

ACKNOWLEDGMENTS

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RECENT ADVANCES IN THE DIAGNOSIS AND DETECTION OF SOME INFECTIOUS DISEASES OF FISHES

G. L. BULLOCK and KEN WOLF[1]

Control of infectious diseases requires rapid and accurate diagnosis as well as detection of latent carriers of disease agents. Effective diagnostic procedures have been available for many of the parasitic diseases of fish, but have been developed only recently for some bacterial diseases and all of the viral diseases. Since morphologic features of parasites are used for diagnosis of disease outbreaks, there has been little need to develop additional diagnostic procedures. The only major parasite disease which requires detection of latent carriers is whirling disease caused by *Myxosoma cerebralis*. New methods of carrier detection have recently been developed and these will be discussed in this paper.

Early diagnostic procedures for bacterial diseases were based on clinical signs and cultural characteristics of the bacteria. Since most of the bacterial diseases of fish are caused by gram-negative organisms, and symptoms and pathology produced by these bacteria are very similar, clinical signs are not reliable in diagnosis of most bacterial fish diseases. Cultural characteristics have been useful in identification of the bacterial pathogens, however, characteristics of pathogens such as *Hemophilis piscium*, the cause of ulcer disease, and *Corynebacterium* sp, the cause of bacterial kidney disease, could not be determined until techniques of culturing were established. With the exception of lymphocystis, diagnosis of the viral diseases was not possible until fish cell culture methods were established, first by Grützner (1958) and later by Wolf et al., (1960).

Diagnostic capabilities for the viral diseases of fish were established with the first isolation of the etiologic agent of infectious pancreatic necrosis (Wolf et al., 1960).

With the present interest in intensive aquaculture, there is increasing need for rapid and accurate methods for disease diagnosis and carrier detection. During the last 10 to 15 years, research has been devoted to improving diagnostic and detection procedures for the major diseases of fishes. The purpose of this paper is to discuss briefly some of the newest methods.

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BACTERIAL DISEASES

Confirmatory diagnosis of bacterial fish diseases has traditionally been based on key morphological and biochemical characteristics of the causative bacterium. For example, the paraphenylendiamine test for tyrosinase suggested by Griffin, Snieszko, and Friddle (1953) has been of value in identification of *Aeromonas salmonicida*, the agent of furnculosis. Morphological properties, enzyme reactions, and differential carbohydrate reactions of the fish-pathogenic bacteria have been used in diagnostic schemes to facilitate identification of the gram-negative pathogens (Bullock, 1964, 1971). However, the present procedures require isolation and purification of the bacterium before identification tests are applied. Selective and differential media allow at least presumptive identification of some gram-negative pathogens without purification or additional diagnostic tests. Commercially available media provide identification of oxidative pseudomonad pathogens, whereas Rimler Shotts (RS) medium (Shotts, and Rimler, 1973) allows identification of members of the *A. liquefaciens* complex.

Organisms in the *A. liquefaciens* complex form yellow colonies on RS medium while other gram-negative pathogens, such as pseudomonads or enteric organisms, form blue, green or colorless colonies.

Serological procedures are now being applied to fish diseases. The identification of *A. salmonicida* can be confirmed by a simple slide agglutination test (Rabb, Cornick, and McDermott, 1964). Similar procedures are available for the agents of columnaris disease (Anacker, and Ordal, 1955), coldwater disease (Pacha, 1968), and Hagerman redmouth disease (Busch, 1973). An indirect fluorescent antibody technique has been used to detect *A. liquefaciens* antibody in fish (Lewis, and Savage, 1972). The latter procedure involves the use of *A. liquefaciens* cells, plasma from fish previously injected with killed *A. lique-*

faciens and rabbit anti-fish serum conjugated with fluorescein isothiocyanate. Serum titers of 1 : 128 were detected within 2 wk after fish were injected, and reached titers of 1: 512 8 wk post injection. Lewis and Savage (1972) suggested that this technique could be used to screen populations of fish for exposure to *A. liquefaciens*. A direct fluorescent antibody technique was used by Lewis and Allison (1971) to detect *A. liquefaciens* cells in the tissues of experimentally infected fish. Globulins from rabbits immunized against five strains of *A. liquefaciens* were pooled and conjugated with fluorescein isothiocyanate and the conjugate applied to tissues of fish infected with *A. liquefaciens*. With this procedure *A. liquefaciens* cells could be detected within 8 hrs after infection and the bacterial cells could be identified even in autoclaved or tissues previously stored in fixatives. Since the conjugated serum would not react with other gram-negative bacteria, the procedure is valuable for diagnostic purposes and detection of low numbers of *A. liquefaciens* cells.

One of the most recently developed fish diagnostic procedures is an immunodiffusion test for corynebacterial kidney disease (KD) of salmonids (Chen et al., 1974). This procedure is particularly useful since it does not require isolation of the slow growing, fastidious *Corynebacterium*. Kidney, spleen, or other material suspected of containing the KD bacterium is used in an Ouchterlony immunodiffusion procedure along with a control KD antigen and rabbit anti-KD serum. If the test material contains the KD bacterium, a band of identity is formed within 24 hr between test and control material. Since strains of the KD bacterium from several geographic locations have been shown to be serologically homogeneous (Bullock, Stuckey, and Chen, 1974), an antiserum prepared against any authentic KD strain can be used reliably to diagnose KD outbreaks. The immunodiffusion procedure is also effective in detecting low level infections.

Because they are sensitive and rapid, serological procedures can also be applied to the detection of latent or asymptomatic carriers of disease agents. Detection of such carriers is often necessary in order to control the spread of infectious disease. The fluorescent antibody techniques of Lewis and Savage (1972) and Lewis and Allison (1971) could be applied to carrier detection as could the passive hemagglutination procedure used by Busch and Lingg (1973) to detect titers in rainbow trout against the Hagerman redmouth bacterium. We use microtiter agglutination procedures to detect antibody in fish serums against the organisms causing furunculosis and kidney disease. Titers of 1: 24 or higher against either pathogen furnishes presumptive evidence that the population has been exposed.

DIAGNOSIS AND DETECTION OF WHIRLING DISEASE

Whirling disease (WD) is one of the most important parasite diseases of salmonid fishes. The parasite attacks cartilaginous tissues of young salmonids and subsequently causes skeletal deformations. The disease probably originated in central Europe and affected brown trout (*Salmo trutta*), but did not produce serious disease in that species (Hoffman, 1970; Hoffman, Dunbar, and Bradford, 1962). Whirling disease was first diagnosed in Germany when rainbow trout (*S. gairdneri*) imported from the United States contracted the disease from brown trout. The parasite has been spread via infected trout into many countries including the United States (Hoffman, 1970). Whirling disease is one of the few myxosporidian diseases universally considered sufficiently serious to warrant control efforts. Since there is no effective treatment for infected salmonids, avoidance of the pathogen is one of the most effective control measures. Diagnosis of clinical cases is based on behavioral changes (whirling, black tail) and identification of *M. cerebralis* spores or trophozoites in histologic lesions, or demonstration of spores in tissues. When fish are lightly infected, detection of spores may be difficult. To detect lightly infected or carrier fish, methods which release and concentrate spores are needed. Several new methods of spore detection have been developed during the last 3 yrs. MacLean (1971) suggested procedures in which defleshed trout heads were ground in a mortar with water and the supernatant dried, stained with malachite green, and examined for spores. Prasher, Tidd, and Tubb (1971) used a high speed blender to grind defleshed skeletal tissues to release spores. They concentrated spores by filtration through cheesecloth and filter paper, and ultimately by centrifugation. Enzymatic digestion of skeletal elements has proven effective in releasing spores (Rydlo, 1971). Trypsin was used by Landolt (1973) to release spores from decalcified bone, while Contos and Rothenbacher (1974) used pepsin to digest mechanically ground tissues. Released spores were concentrated and partially purified by centrifugation with ether (Landolt, 1973) or filtration through a 72 μ sieve followed by centrifugation (Contos, and Rothenbacher, 1974).

Markiw and Wolf (1974a) developed a procedure using both trypsin and pepsin digestion for spore release followed by centrifugation through dextrose. Gill arches and skeletal elements were first treated with pepsin then further digestion was effected with trypsin. Spores released by the digestion were then

concentrated by centrifugation. Pelleted spores and debris were separated by differential centrifugation through 55% dextrose. In that process, spores were driven through and concentrated at the bottom of the dextrose while the debris remained at the top. The procedure has proven effective in detection of low numbers of spores. The complete technique, using both enzymatic digestions and centrifugation through 55% dextrose has been shown to be 10.5 times more effective in detecting low numbers of spores than examination of mechanically ground skeletal elements (Markiw, and Wolf, 1974b).

The use of these new procedures for spore release and detection offers a highly effective means of controlling WD by providing sensitive detection of lightly infected fish.

VIRAL DISEASES

In diagnostics of viral diseases noteworthy advances made during the last several years may be grouped in three categories. One covers the introduction of and advancement in applications of microculture techniques for viral titrations and serological identifications. A second has been the successful development and use of fluorescent antibody techniques (FAT) for diagnosis and detection of Egtved virus, the cause of viral hemorrhagic septicemia (VHS) and infectious pancreatic necrosis virus (IPNV). The third group of advances concerns plaque assay procedures that are used for serological identifications and for rapid presumptive diagnoses that can approach serology in accuracy.

Microcultures, or the so-called microtiter procedures, are a relatively new introduction to fish viral diagnostics and identification. A common form of the plate is a 12.5cm×8cm plastic block containing 96 flat-bottom 6mm diameter wells each of which has a capacity of about 0.3 ml. Gratzek et al. (1973) were the first to give details of a specific use in fish virology; they used the microculture technique to quantify channel catfish virus (CCV) and its corresponding antibody. Relevant to our presentation, the microculture technique offers decided savings in time, reagents, and space. As an example, less than 10 ml of cell suspension are needed for seeding the 96 wells. In addition, the plates may be stained and kept to provide a permanent record. Perhaps the greatest value of all is speed; because plates may be seeded simultaneously with virus or virus-serum mixtures and cells, the need to grow cultures to confluency before inoculation is obviated. The saving of time usually amounts to 2 or 3 days.

In addition to CCV, microculture systems have been used for serological identification in the diagnoses of infectious hematopoietic necrosis (IHN), infectious pancreatic necrosis (IPN), and viral hemorrhagic septicemia. There are no known reasons why microculture procedures cannot be used for diagnoses of all fish viral diseases. The reader is cautioned, however, that each well in a microculture plate has only about 1.0 cm^2 of cell growth area, and that cultures of greater area offer greater sensitivity in detecting viruses.

Fluorescent antibody techniques employ fluorescein isothiocyanate or related fluorescent compounds conjugated with immune serum globulins to provide a microscopically visible serological reaction between homologous reagents. Argot (1969) first employed the procedures in an unpublished portion of her dissertation on IPNV. Jørgensen and Meyling (1972) reported that FAT was a rapid and sensitive method of diagnosis of acute cases of VHS and that heart was the preferred tissue. They qualify their findings by noting that among 11 chronic cases of VHS, negative with FAT, three yielded virus isolations. Jørgensen and Meyling (1972) also used FAT successfully with IPNV and noted that these two viruses did not cross-react in the FAT.

In another report, Jørgensen (1972) noted the existence of two serological groups of Egtved virus and shows that both reacted with the conjugate prepared with Group I antiserum. In contrast, the Group I conjugate failed to react with one isolant each of IPNV, IHNV, and the agent of spring viremia of carp (SVC). In the same work, the anti-IPNV conjugate reacted only with its homologous virus, but other IPNV isolants were not tested. The results clearly show the specificity of the reaction and its value in fish viral diagnostics.

Piper, Nicholson, and Dunn (1973) used a direct FAT with three isolants of IPNV. They found that the brightest reaction occurred when the virus-antibody preparation was homologous and that one heterologous strain of IPNV fluoresced less strongly and another not at all. The results indicate that with IPNV, direct FAT with monotypic serum could give an arroneous diagnosis if a heterologous strain of IPNV was encountered. In principle, the weakness of direct FAT for IPNV could be avoided either with an indirect procedure or with polyvalent antiserum such as was used for neutralization tests by Lientz and Springer (1973). Tu, Spendlove, and Goede (1974) reported in detail their work with direct FAT using 11 North American isolants of IPNV and a pool of antiserums prepared against the agents. Of nine IPN viruses tested, all fluoresced with the antiserum, but IHNV did not. In addition, they found FAT to be about three times more sensitive than plaque assay.

Like Piper, Nicholson, and Dunn (1973) they recommend that cell cultures should be stained between 10 and 12 hr post-infection.

Plaque assay, one of the procedures employed for visualizing the effects of viruses in susceptible cell cultures, has been used in fish virological research for at least 5 yr. McCain, Fryer, and Pilcher (1971) used plaque assay in serum neutralization tests which form a basis for identifying IHNV. In the same laboratory, McAllister, Fryer, and Pilcher (1974) used plaquing to show that there was no cross-neutralization between IHN and Egtved viruses and that each agent could be serologically identified with its homologous antiserum. Jørgensen (1974) used plaque assay procedures in serum neutralization tests to distinguish between IHNV, and the rhabdovirus of acute infectious dropsy (AID) or SVC. In the same comprehensive review of fish virology in Denmark, Jørgensen (1974) also distinguished between serological kinds of IPNV. Bachmann and Ahne (1974) similarly used plaque reduction neutralization tests and differentiated between the rhabdoviruses of VHS, IHN, and the agent of SVC; perhaps most importantly, they provided serological evidence which showed a common etiology for SVC and swimbladder inflammation of carp. The work of de Kinkelin and Berre (1974) also employed plaque assay, and the authors concluded that SVC and swimbladder inflammation of carp had a common etiology.

Plaque assay procedures are a firm part of fish viral diagnostics, because the assay methodology is acknowledged to be the most accurate means of quantifying infectivity. When critical identifications are needed, serological tests of plaque reduction promise to continue as a standard method.

Fish virology is currently in a highly favorable position because plaque assay has been shown to provide a rapid presumptive—nevertheless highly accurate—identification of the problem viruses of salmonids. Moreover, the procedures give results which distinguish between two viruses if more than one is present.

Wolf and Jørgensen (1970) noted that neutral red staining of RTG-2 cells infected with Egtved or IPNV showed differences in the resulting plaques. Wolf and Quimby (1973a, b) determined procedures whereby all fish viruses could be plaqued in normal atmosphere with a single method and that the unified method gave preparations that could be fixed and stained with crystal violet. In RTG-2 cells, the plaques of Egtved, IHNV, and IPNV were characteristically distinct—so much so that presumptive diagnoses approach serological identifications in accuracy. Similarly, these authors describe the salient features of plaques of five other fish viruses thus far isolated and suggest how with epizootiological and clinical data the various fish virus infections can be presumptively diagnosed with accuracy.

The significance of plaque characteristics as a diagnostic tool is enhanced because the procedures will show evidence for a second virus if it is present. Similarly, Wolf and Quimby (1973a) reported two kinds of IPNV plaques; Type I (as represented by reference strain VR 299) was relatively lytic and produced plaques (2–3 mm) in 2 days at 20°C. Type II plaques were less destructive and required 3 days to reach comparable size.

EASTERN FISH DISEASE LABORATORY IMPLEMENTATION OF FISH DIAGNOSTICS

Recognizing the universal need for standard reagents in fish disease diagnostics, the Eastern Fish Disease Laboratory has begun a program of preparing and standardizing antiserums against problem bacterial and viral diseases. Thus far, these antiserums have been prepared and distributed: IPN, CCV, IHN, KD, *A. salmonicida,* and the Hagerman redmouth organism. It is our intention to make reference quantities of such reagents available to researchers everywhere and diagnostic quantities available to Federal and State fish health workers in North America.

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IMMUNIZATION OF FISH FOR THE CONTROL OF VIBRIOSIS

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ABSTRACT

Efficacious vaccines were developed for the control of *Vibrio anguillarum*, the etiological agent of vibriosis in salmonid fish. These bacterins can be administered either orally or parenterally. It was determined that both formalin-killed lyophilized whole cells and wet-packed whole cells of the organism are effective oral immunogens. Intraperitoneal injection of 0.1 ml containing 2×10^8 formalin-killed bacterial cells suspended in saline and mixed with Freund complete adjuvant is capable of providing protection to fish exposed to natural challenge with *V. anguillarum*.

Several parameters under which the oral vaccine can be effectively used were examined. These investigations revealed that protection is provided to fish vaccinated for 15 days with a ration containing 0.5 mg of the wet whole cell vaccine per gram. Increasing the number of days the vaccine was fed to as many as 45 days did not increase the degree of resistance in immunized fish. Decreased mortality was also not observed in groups of fish fed a diet containing higher concentrations of vaccine. Other studies demonstrated that oral immunization of fish can be successfully accomplished at water temperatures ranging from 4 to 21 C.

INTRODUCTION

The increased interest in aquaculture has demanded a concern for fish diseases and methods for their control. Fish cultural management techniques designed to decrease environmental stress and thus reduce the incidence of disease, and the use of chemotherapy are two methods which have been employed in controlling infectious diseases of fish. Each of these methods, however, has inherent problems when animals are intensively cultured in marine or estuarine environments. For several years our laboratory has been directed toward the development of effective vaccines which might overcome the limitations of chemotherapy and management techniques. Vaccines against several fish pathogens have been explored including bacterins which are effective in controlling *Vibrio anguillarum*, the causative agent of vibriosis. *Vibrio anguillarum* has been responsible for devastating losses among numerous species of fish, and its distribution seems to be world-wide. The number and frequency of reports of vibriosis indicate the economic importance of this disease among food fish.

The etiological agent, *V. anguillarum*, is a gram-negative, slightly curved rod, which is motile by a single polar flagellum. These bacteria are anaerogenic fermentors of glucose and certain other sugars; they are oxidase and catalase positive and have a sodium chloride requirement for optimal growth. Members of the genus are sensitive to 0/129 (2, 4, diamino, -6, 7-di-iso-propyl pteridine) and to novobiocin.

Pathological symptoms in salmonids which are associated with *V. anguillarum* include: erythema at the base of the fins and around the mouth, inflamation and reddening around the vent, red necrotic lesions of the abdominal musculature and petechiae on the body surface. In more advanced stages of the disease, ulcerative hemorrhaging and septicemia occur. The causative agent can be readily isolated from the kidney of diseased animals. Similar symptoms have been reported in other species of fish, although not always as well defined. Gross pathological symptoms are also less apparent in acute cases of vibriosis in salmonid fish.

Research has been done in the development of vaccines which may be effective in the control of this disease. Because oral administration of any bacterin used in large populations of fish is the most efficient method of delivering the vaccine, the major portion of our investigations have been in exploring oral vaccines and the conditions under which they can be effectively used. In addition to oral immunogens, a parenterally administered bacterin has been investigated. The preparation of each antigen is outlined in Figure 1. The procedure for mass culturing of the bacterial cells was the same in the preparation of the oral bacterins. Ten ml of broth were inoculated with organisms from a stock culture of *V. anguillarum* and

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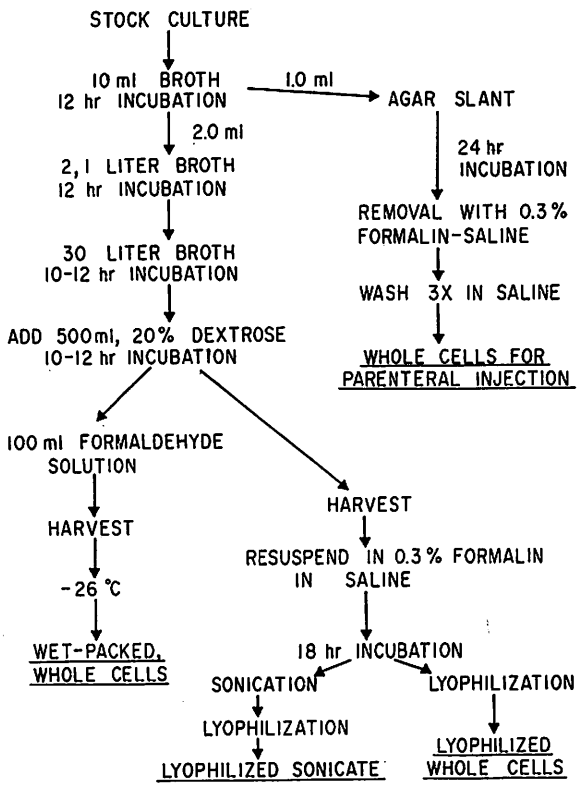

Figure 1. Methods used for vaccine preparations.

after 12 hours incubation two ml of this broth culture were used to inoculate each of two, one-liter quantities of broth which were incubated 12 hours. These two liters of broth culture were used as the inoculum for 30 liters of broth which had been sterilized in a fermenter. After an incubation period of 10–12 hours, 500 ml of a 20% dextrose solution was added and then the culture was allowed to incubate for an additional 12 hours. For the preparation of lyophilized whole cells or the lyophilized sonicate, the cells were harvested by high speed, continuous flow centrifugation. Two hundred-fifty grams of the harvested cells were then resuspended in one liter of a saline solution containing 0.3% formaldehyde and mixed for 24 hours. The cells were then sonically disrupted and lyophilized, or liophilized without being ultrasonically treated. For the preparation of the wet-packed, whole cell vaccine, the bacterial cells were killed in the fermenter by the addition of 100 ml formaldehyde solution directly into the 30 liter broth culture. The cells were harvested after one hour then frozen and stored at −26C.

Vaccines were also prepared for parenteral administration. Because smaller quantities of the bacterin were needed for this type of immunization, the *V. anguillarum* cells were cultured on agar surfaces. This was done by preparing agar slants in eight ounce prescription bottles which were inoculated with one ml of a 12 hour broth culture of the desired organism. After incubation, the bacteria were removed from the agar surface with 0.3% formalin-saline solution. The cells remained in this solution for one hour and were then washed three times by centrifugation in phosphate buffered saline.

The method used for oral vaccination followed a similar procedure in all experiments conducted. Fish, either chinook salmon (*Oncorhynchus tshawytscha*) or coho salmon (*O. kisutch*), were fed a diet containing the bacterin incorporated at some selected level per gram of ration. This diet was administered to fish held at a fresh water facility that was furnished with pathogen-free well water at an ambient temperature of 12C. There was also the capability of varying the water temperature in experimental aquariums from 4 to 23C.

Efficacy of the bacterins was tested by exposing vaccinated fish to a natural challenge of *V. anguillarum* at a salt water rearing impoundment at Lint Slough on the Oregon Coast. At this facility, groups of experimental animals were held in one-meter diameter fiberglass tanks which were furnished with salt water. *Vibrio anguillarum* is endemic at this rearing impoundment and reaches epizootic proportions in the warmer months of the year when the water temperature rises to above 12C.

A necropsy was performed on all experimental animals which died during the challenge. The animals were examined for gross pathological symptoms, dissected aseptically, and bacteriological cultures were then prepared from kidney tissue using an agar medium. After incubation, the plates were examined for typical colonies of *V. anguillarum*. Presumptive tests on these cultures included microscopic examination using the Gram reaction, and also morphology and motility using phase contrast microscopy. Infection by *V. anguillarum* was confirmed with rapid slide agglutination tests using the suspected isolates as antigens and *V. anguillarum* antiserum which had been prepared in rabbits.

EXPERIMENTAL RESULTS

The original vaccine formulated and tested in our laboratory was a lyophilized sonicate of *V. anguillarum*. The organisms were sonically disrupted in an effort to expose as many cellular antigens as possible and then lyophilized to facilitate storage and handling. This bacterin was fed to a group of chinook salmon held in fresh water. When exposed to the disease causing organism in salt water, it was found that the mortality in the vaccinated lot was suppressed as compared to untreated control animals (Figure 2).

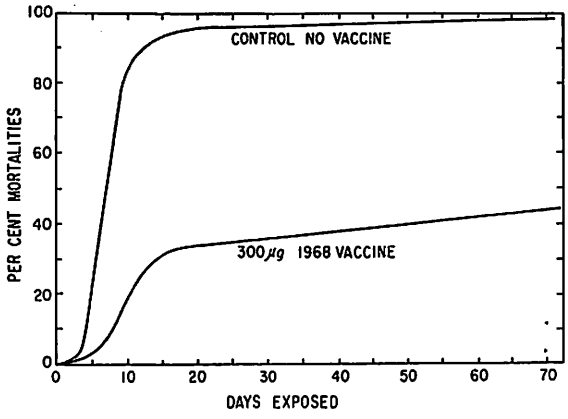

Figure 2. Effect of oral vaccine for control of vibriosis in spring chinook salmon.

The goals of further experimentation were two-fold. First, to determine whether the efficacy of immunization could be improved by altering the vaccine, and second to produce a more economical bacterin which would be as effective as the lyophilized sonicate used in the original studies. Three preparations of oral bacterins were employed. These were the lyophilized sonicate and lyophilized whole cells; both of these had been formalin-killed. Also included was a lyophilized sonciate which was not treated with formalin.

Each of the oral vaccines was incorporated into the fish's ration at a level of 2mg of vaccine/gm of diet. Groups of 200 spring chinook salmon (mean weight 3.8 gm) were fed 30 gm of diet/200 fish per day. Groups of unvaccinated control fish received a diet containing no vaccine. These different preparations were fed for 30 days. After the vaccination period, all fish were maintained in fresh water for 15 days before all groups of fish were simultaneously exposed to the natural challenge of *V. anguillarum* at Lint Slough and were continually challenged for 30 days.

Formalin-killing was omitted in one preparation to determine if that treatment had any adverse effects on the protective antigens of the cell. The necessity of exposing antigens by sonic disruption was tested by preparing a lyophilized whole cell vaccine. After challenge to *V. anguillarum*, total mortality among vaccinated lots of fish ranged from 7 to 11%, while the unvaccinated control experienced 70% mortality (Table 1). Results obtained indicated that all methods of vaccination elicited some degree of protection when compared to the unvaccinated control group. These data showed that formalin is not detrimental to protective antigens and that sonication is unnecessary in the preparation of an efficacious vaccine.

In a subsequent vaccine comparison, the lyophilized whole cell bacterin was fed to a group of 200 fall

Table 1. Efficacy of three oral bacterins for control of vibriosis in spring chinook salmon.

Bacterin administered[1]	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Formalin-killed Lyophilized Sonicate	200	24	21	11
Formalin-killed Lyophilized Whole Cells	200	14	14	7
Lyophilized Sonicate No Formalin Treatment	200	15	13	7
Unvaccinated Control	200	147	140	70

[1] Fed at a level of 2 mg/gm of Oregon Test Diet for 30 days followed by a 15 day post-vaccination period in fresh water.
[2] Mean weight 3.8 gm/fish.
[3] After 40 days days natural challenge to *Vibrio anguillarum* in salt water.

Table 2. Efficacy of two oral vaccines, lyophilized whole cells and wet-packed whole cells, for control of vibriosis in fall chinook salmon.

Bacterin administered	Number of fish/group[3]	Total number of deaths[4]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Lyophilized Whole Cells[1]	200	19	14	7
Wet-packed Whole Cells[2]	200	16	12	4
Unvaccinated Control	200	115	110	55

[1] Fed at 2 mg/gm of Oregon Test Diet for 45 days.
[2] Fed at 10 mg/gm of Oregon Test Diet for 45 days.
[3] Mean weight 0.3 gm/fish.
[4] After 40 days natural challenge to *Vibrio anguillarum* in salt water.

chinook salmon (mean weight 0.3 gm). The efficacy of this preparation was compared to wet whole cell vaccine which was fed to a similar group of fish. The lyophilized preparation was incorporated into the diet at a level of 2 mg of vaccine/gm of ration. Dry weights were determined on ten batches of vaccine and it was demonstrated that the wet-packed preparation contained approximately 80% water. Therefore, this bacterin was incorporated at a level of 10 mg of vaccine/gm of ration. Each group of fish was fed one of these diets for 45 days while a control group was fed no vaccine. After the vaccination period, the animals were maintained in fresh water for 15 days before being transported to Lint Slough where they were challenged for 40 days. The unvaccinated fish died at a level of 55%, while the groups of animals which had received the vaccines had mortalities of 6 and 7% (Table 2). Although in some instances lyophilized preparations may be more convenient to work with, an effective vaccine can be made simply by growing the cells in mass culture, killing them and then harvesting the organisms.

In later experiments, some parameters under which these vaccines could be employed effectively were investigated. The first condition to be studied was the period of time the vaccine was administered. Fish were orally vaccinated for different lengths of time to determine whether an increased number of vaccine dosages would result in increased protection when the animals were challenged. The lyophilized whole cell vaccine was added to the diet at a level of 2 mg of vaccine/gm of ration and fed to groups of 200 fall chinook salmon (mean weight 0.3 gm). One group of animals was administered this diet for 15 days, one for 30 days, and one for 45 days. The vaccination periods were begun so that each experimental group received its last vaccine dosage on the same day. All immunized fish and an unvaccinated control group were then maintained in fresh water for 15 days before being simultaneously challenged for 40 days at Lint Slough.

When the length of the vaccination period was varied, it appeared that increasing the time of vaccine administration to more than 15 days did not appreciably increase the protection elicited to experimental animals (Table 3).

Another experiment was done with a similar design. In this study, lots of 100 coho salmon (mean weight 15 gm) were fed a diet containing 2 mg of wet whole cell vaccine/gm of ration. The vaccination

Table 3. Efficacy of selected vaccination periods for the control of vibriosis in fall chinook salmon

Vaccination period[1] (days)	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
15	200	30	21	12
30	200	30	25	13
45	200	41	32	16
0 (Unvaccinated Control)	200	170	168	84

[1] Vaccine fed at a level of 2 mg of lyophilized whole cell bacterin/gm of Oregon Moist Pellets.
[2] Mean weight 0.3 gm/fish.
[3] After 40 days natural challenge to *Vibrio anguillarum* in salt water.

Table 4. Efficacy of selected vaccination periods for the control of vibriosis in coho salmon.

Vaccination period[1] (days)	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
15	100	0	0	0
10	100	5	4	4
5	100	7	7	7
2	100	13	12	12
1	100	13	13	13
0 (Unvaccinated Control)	100	27	26	26

[1] Vaccine fed at a level of 2 mg of wet whole cell bacterin/gm of Oregon Moist Pellets.
[2] Mean weight 15 gm/fish.
[3] After 20 days natural challenge to *Vibrio anguillarum* in salt water.

periods studied were 1, 2, 5, 10, and 15 days. After immunization, all groups were maintained one week in fresh water before being challenged at Lint Slough for 20 days.

Data collected from these experiments in which the vaccination periods were less than 15 days, indicated that decreasing the number of dosages did affect the protection provided by the vaccine. Although some degree of resistance was detected in groups of animals fed for one or two days, fish which received the vaccine for longer periods of time were better protected (Table 4).

The next parameter which was studied was the concentration of vaccine which was incorporated into the diet. As in the preliminary tests with feeding periods, an experiment was done to determine if greater concentrations of vaccine would elicit a higher degree of resistance.

Groups of 200 fall chinook salmon (mean weight 6.5 gm) were fed wet whole cell vaccine at levels of 2, 5, or 10 mg/gm of diet for 30 days. The fish remained in fresh water for 15 days and were then challenged at Lint Slough for 30 days.

The animals which were fed 2, 5, or 10 mg of vaccine/gm of ration experienced mortalities due to vibriosis of 25, 19, and 18% respectively. Sixty-six percent of the unimmunized fish died of vibriosis (Table 5).

Because the results from this experiment indicated that increasing vaccine concentration did not increase protection, a subsequent study was undertaken to test lower vaccine concentrations. In this study, lots of 100 coho salmon (mean weight 12.6 gm) were fed 0.5, 1, 2, and 5 mg of the wet whole cell vaccine/gm of ration. The vaccine was fed for only 15 days, followed by 5 days in fresh water on a diet without vaccine. The fish were then exposed to *V. anguillarum* for 20 days at the salt water rearing facility.

The unvaccinated control group had a 22% mortality, while groups receiving 0.5, 1, 2, or 5 mg of vaccine/gm of diet experienced losses of 1 to 2% (Table 6). Although the lower limits of vaccine concentration were not reached, results of this experiment indicate that a very small amount of antigen is necessary to stimulate the immune response in these animals.

Variations in water temperatures are encountered among hatchery locations and with the seasons of the year. It has been reported that agglutinating antibody production in poikilothermal animals is slower

Table 5. Efficacy of selected vaccine concentrations for control of vibriosis in fall chinook salmon.

Vaccine concentration administered[1]	Number of fish/group	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
2	200	55	50	25
5	200	42	37	19
10	200	38	36	18
0 (Unvaccinated Control)	200	139	138	66

[1] Mg of vaccine/gm of Oregon Test Diet fed for 15 days.
[2] Mean weight 6.5 gm/fish.
[3] After 30 days natural challenge to *Vibrio anguillarum* in salt water.

Table 6. Efficacy of selected vaccine concentrations for the control of vibriosis in coho salmon.

Vaccine concentration administered[1]	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
0.5	100	1	1	1
1.0	100	2	2	2
2.0	100	8	4	4
5.0	100	6	2	2
0.0 (Unvaccinated Control)	100	24	22	22

[1] Mg of vaccine/gm of Oregon Test Diet fed for 15 days in fresh water.
[2] Mean weight 12.6 gm/fish.
[3] After 20 days natural challenge to *Vibrio anguillarum* in salt water.

at low temperatures. Because all of the immunization experiments that have been described here had been conducted in water temperatures of 12C, it was desirable to determine what effect diverse water temperatures had on the immune response of animals which were orally vaccinated.

Table 7. Efficacy of oral vaccination of coho salmon held at selected water temperatures.

Temperatures at which fish were vaccinated[1]	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
3.9	100	6	0	0
6.7	100	3	0	0
9.5	100	4	0	0
12.2	100	9	1	1
15.0	92	19	5	5
17.8	100	8	0	0
20.6	100	5	1	1
12.2 (Unvaccinated Control)	100	44	37	37

[1] Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a 14 day tempering period.
[2] Mean weight 6.5 gm/fish.
[3] After 20 days natural challenge to *Vibrio anguillarum* in salt water.

Table 8. Efficacy of oral vaccination of coho salmon held at selected water temperatures

Temperatures at whichs fish were vaccinated[1]	Number of fish/group[2]	Total number of deaths[3]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
3.9	75	2	1	1
	100	13	5	5
6.7	95	4	2	2
	60	4	1	2
9.5	97	0	0	0
	100	6	2	2
12.2	90	2	0	0
	80	6	0	0
15.0	96	1	1	1
	100	0	0	0
17.8	97	0	0	0
	86	1	0	0
20.6	99	0	0	0
	87	0	0	0
12.2	100	72	72	72
(Unvaccinated Control)	100	86	83	83

[1] Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a 7 day tempering period.
[2] Mean weight 3.3 gm/fish.
[3] After 20 days natural challenge to *Vibrio anguillarum* in salt water.

Table 9. Comparison of the efficacy of parenteral and oral administration of vaccine for the control of vibriosis in spring chinook salmon

Method of vaccine administration	Number of fish/group[3]	Total number of deaths[4]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Oral[1]	200	11	7	4
Injected Intraperitoneally[2]	150	5	4	3
Unvaccinated Control	200	147	144	72

[1] Vaccine was lyophilized whole cells incorporated into Orgon Test Diet and fed at a level of 2 mg of vaccine/gm of diet for 30 days.
[2] Vaccine was 0.1 ml of a Freund adjuvant-saline suspension containing 2×10^8 cells.
[3] Mean weight 3.8 gm/fish.
[4] After 40 days natural challenge to *Vibrio anguillarum* in salt water.

Table 10. Comparison of the efficacy of parenteral and oral administration of vaccine for the control of vibriosis in fall chinook salmon

Method of vaccine administration	Number of fish/group[3]	Total number of deaths[4]	Number of deaths caused by vibriosis	Percent mortality caused by vibriosis
Oral[1]	200	26	19	10
Injected Intraperitoneally[2]	150	13	10	7
Unvaccinated Control	200	172	160	80

[1] Vaccine was liophilized whole cells incorporated into Oregon Test Diet and fed at a level of 2 mg of vaccine/gm of diet for 30 days.
[2] Vaccine was 0.1 ml of a Freund adjuvant-saline suspension containing 2×10^8 cells.
[3] Mean weight 23.0 gm/fish.
[4] After 40 days natural challenge to *Vibrio anguillarum* in salt water.

Lots of coho salmon (mean weight 6.5 gm) were tempered from 12C to each of seven temperatures, to include: 3.9, 6.7, 9.5, 12.2, 15.0, 17.8, and 20.6C. The Oregon State University Fish Disease Laboratory is designed to record in degrees Fahrenheit, and these temperatures correspond to 39, 44, 49, 54, 59, 64 and 69 F.

For 15 days these animals were fed a diet containing 2 mg of wet whole cell vaccine/gm of ration. After the fish were administered the vaccine, they were tempered back to 12C. Tempering of fish took place during a one-week period at temperature increments of 3C every two days. After all groups had been returned to 12C, they were maintained at this temperature for one week before being exposed to *V. anguillarum* at Lint Slough. The vaccinated fish were compared to a similar unimmunized control group which had been held at 12C throughout the freshwater phase of the experiment (Table 7).

The experiment was repeated with three modifications of the design described above. First, the fish used in this experiment were smaller (mean weight 3.3 gm), therefore, these animals consumed less diet than those used in the previous study. Secondly, replicate groups of animals were employed. The third modification of the experimental design was that after the diet containing vaccine was fed for 15 days, each group of fish was held at its respective temperature for one week before being tempered back to 12C. The fish were challenged naturally at Lint Slough for 20 days.

All immunized groups had mortalities of less than 5% while control animals experienced mortalities as high as 83% (Table 8). The results of these experiments indicate that diverse water temperatures do not preclude oral immunization with bacterins prepared for the control of *V. anguillarum*.

An experiment was designed to determine whether parenteral administration of vaccine would provide protection to fish and to compare the efficacy of this method to oral immunization. In these studies two types of fish were used, spring chinook salmon (mean weight 3.8 gm) and fall chinook salmon (mean weight 22.7 gm). Experimental groups consisted of 200 animals in the orally vaccinated groups and 150 fish in the groups which were injected.

The oral bacterin used in this experiment was the lyophilized whole cell vaccine which was incorporated into a diet at a level of 2 mg/gm of ration. The spring chinook were fed 30 gm of diet/200 fish per day, while the fall chinook received 100 gm of diet/200 fish per day. The duration of oral vaccination was 30 days followed by a 15 day post-vaccination period.

Fish which were parenterally vaccinated were injected intraperitoneally with 0.1 ml of a Freund adjuvant-saline suspension of cells. Both types of fish received the same dosage (approximately 2×10^8 cells/animal) and were vaccinated 50 days prior to the time they were challenged. Throughout this period in fresh water, these animals were maintained on a ration containing no vaccine.

The orally and parenterally vaccinated fish were simultaneously exposed for 40 days to natural challenge with *V. anguillarum*. The mortality of these animals was compared to similar groups of fish which had not received any vaccination treatment.

Each vaccinated group showed little variation in mortality (Table 9). The immunized spring chinook showed losses of 3% in the injected group and 4% in the orally immunized lot. The unimmunized control animals had a 72% mortality. Similar results were obtained from the experiment using fall chinook (Table 10). Injected fish had a 7% mortality; the orally vaccinated group showed a 10% loss, and the control animals experienced an 80% loss. These results indicated that both methods of vaccine administration can be effective in the control of vibriosis.

Effective vaccines against *V. anguillarum* have been developed and have been tested intensively

under laboratory conditions. Although there are inherent problems in the immunization of fish on a production scale, the vaccine developed during this study offers a potentially successful method for the control of vibriosis of fish in marine aquaculture.

VIBRIO INFECTION OF MARINE FISHES

JIRO TANAKA[1]

INTRODUCTION

In 1909 Bergmann recognized that many fish diseases in various species throughout the world are caused by an infection of *Vibrio anguillarum*. Since then, Vibrio infections have been identified and studied in many countries. In Japan, Kusuda (1965) investigated Vibrio diseases in sunh species as the Yellowtail (*Seriola quinqueradiata*); Parrot Bass (*Oplegnathus faciatus*); Sweet Fish (*Plecoglossus altivelis*); and in 1969 Egusa made a review of the literature on the fish diseases caused by *Vibrio anguillarum*.

More recently, Vibrio related diseases have frequently occurred in the cultured fry of marine fishes such as the Yellowtail and Red Sea Porgy after the fry have been transferred into rearing ponds or floating pens. Last year fresh-water reared smelt fry were also attacked by Vibrio bacteria.

SYMPTOMS IN JAPANESE MARINE FISHES

The symptoms vary depending upon the species, but in general, the first symptom to appear is hemorrhaging from the fins (especially the dorsal, pectoral, and caudal fins). In later stages the hemorrhaging spreads over other body surfaces, particularly the bases of the fins and the area around the anus. In some species red spots may appear on the fins and the body surface. Other externally visible symptoms are hemorrhages on the eyelids and inside the eye, the cornea may become clouded, the anus may become red and the opening enlarged, and the gills may appear deprived of blood.

Internally, hemorrhaging may also be visible on the intestines, liver, spleen, airbladder, kidneys, internal membranes, etc. Usually the intestines, liver, and kidney are damaged most, and in extreme cases these organs become ulcerated. *Vibrio anguillarum* causes hemorrhaging in all infected tissues. It is believed that infected fish eventually die of pernicious anemia.

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CHARACTERISTICS OF THE BACTERIUM

Characteristically, *Vibrio anguillarum* is a slightly curved, gram negative bacillus with a single apical flagellum. Sometimes 2 bacteria will be joined but never more than 2. In length the bacteria normally measure 0.5 to 1.5μ; but after successive generations of culture, some bacteria enlage to 4.0 to 5.0μ and lose their curved shape. In some cultures there may be many deformed bacteria that are enlarged or round in shape.

A number of authors have reported on the culture of *Vibrio anguillarum* and it appers there are several strains of the bacteria which react differently to such tests as the Voges-Prokauer test, Indole Production, and to acids from sucrose, arabinose, galactose, cellobiose, starch, mannitol and inositol. These apparent strains are possibly caused by the different culturing techniques used by different researchers, or to significant changes in the bacteria after several generations of culture.

THE RELATIONSHIP BETWEEN GROWTH AND TEMPERATURE

The various strains of *Vibrio anguillarum* grow at different rates at any particular temperature. From the results of many experiments, Muroga (1967) determined that growth may occur in the temperature range of 8 to 37°C. However, if *Vibrio anguillarum* obtained from a marine fish is cultured in a madium containing 0.5% NaCl, the temperature range is only from 20 to 30°C; and in a medium containing 3.0% NaCl, the range increases (20 to 37°C). The optimum temperature for *Vibrio anguillarum* obtained from marine fishes is about 25°C.

THE RELATIONSHIP BETWEEN GROWTH AND PH

Vibrio anguillarum will grow in a very wide range of PH's, from PH 6 to 10, but the optimum PH for

growth of *V. anguillarum* from marine fishes lies between 8.0 and 8.5.

THE RELATIONSHIP BETWEEN GROWTH AND SALINITY

At a constant pH of 8.0, *V. anguillarum* taken from saltwater reared fish showed no growth when cultured in a medium containing no NaCl. Media containing 0.5 to 6.0% NaCl were required for growth to occur with optimum growth occurring in the range of 2.0 to 4.0% NaCl. Muroga (1967) reported that *V. anguillarum* from freshwater reared smelt grew in a media with 0.07 to 7.0% NaCl. This same wide range had previously been observed by Shäperclaus, Nybelin, Smith and Ross; and it is believed that several varieties may be involved. Many unanswered questions remain concerning both the adaptation of the bacteria to diffent salinities, and the method of transmission of the diseases.

PREVENTION AND TREATMENT

An effective way of preventing outbreaks of Vibrio infection in marine fish is to immerse fry in a solution of Nitrofran Debris before transferring them to rearing ponds or floating pens. Treatment of diseased fish may be accomplished by feeding Anti-biotex, Sulphonamide, or Nitrofran Debris.

I have used both the preventative bath and the feeding treatment with yellowtails. An oral dose of Sulphomonometoxin was used in the treatment of infected yellowtails, and the concentration of the drug in the blood and internal organs was determined by bio-assay, chemical assay, and bioautography. The table shows the results of feeding 100mg/1kg B. W. and 200mg/kg B. W. It can be seen that the drug was absorbed rapidly, and then the amount of drug began to decrease quickly in just 4 to 6 hours. This trend is seen in the blood, the plasma, the liver, kidney, spleen, and muscle tissue. The drug concentration was higher for fish that received that received the dosage of 200mg/kg B. W. Therefore, it apears that yellowtails should be given 200mg/kg B. W. on the first day of treatment and 100mg/kg B. W. on succeeding treatment days.

Of course, the amount of drug appropriate for each fish species must be determined experimentally. Also, because the bacteria will develop a resistance to the drug over a period of time, vaccines may be a better treatment. Consequently, I am looking foreward to Dr. Rohevec's talk on immunization.

In conclusion, I would like to thank Dr. Egusa for his advice on this project, and also for slides that he loaned me for this talk.

BACTERIAL TUBERCULOIDOSIS OF CULTURE YELLOW TAIL

TOSHIHIKO MATSUSATO[1]

Many diseases of fishes on mariculture have been observed in Japan and are becoming a serious constraints for the progress. (Figure 1) The bacterial tuberculoidosis of the yellow tail (hamachi) is one of the most serious diseases of the culturists. This disease was first reported by Kubota, Kimura, Kusuda and Egusa in 1970. It occurs seasonally at almost all hamachi culturing farms. In 1972, Kusuda determined the pathogen of this disease as *Pasteurella piscicida.*

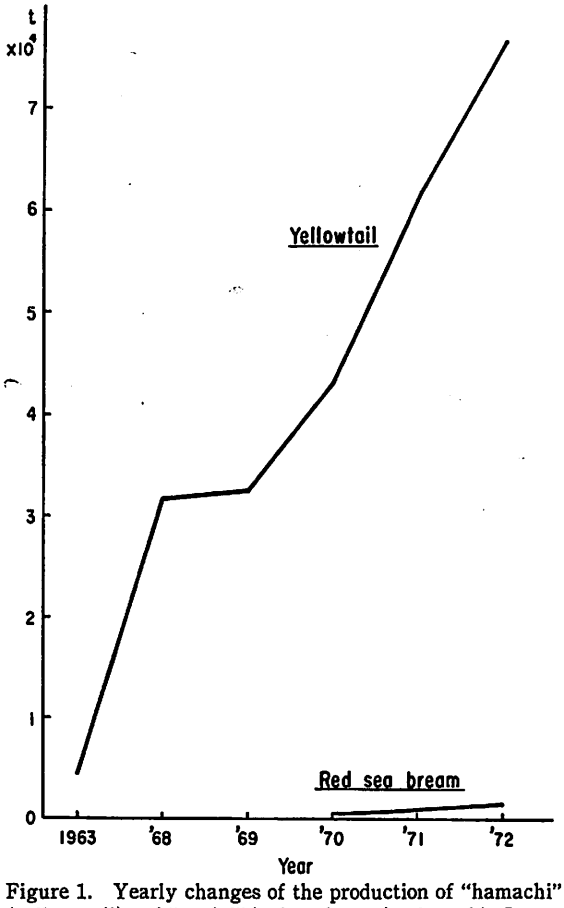

Figure 1. Yearly changes of the production of "hamachi" (yellow-tail) and "madai" (red sea bream) cultured in Japan.

The purpose of this report is to review to pertinent infomation on this disease.

HISTORY

In the early summer of 1964, a new bacterial infectious disease being characterised tubercula- like granules occured in kidney and spleen of infected fish at the hamachi culturing farms along the coasts of Miyazaki and Kochi pref..

In 1968, the disease was widely spreaded in the south-west coast of Japan.

In 1969, the disease carried out serious mortality in young hamachi at almost of the farms.

In 1970, numerous outbreaks of this disease were occured, and result in serious losses were reported.

Since 1971, serious prevalence of the disease has been observed once or twice a year.

This disease was originally discribed by Kubota, Kimura and Egusa in 1970. They provided an excellent description from mainly histpathological viewpoint and named "bacterial tuberculoidosis." The pathogenic bacterium isolated from tubercula-like granules of infected fishes was classified by Kusuda in 1970, and the name *Pasteulla seriola* sp nov was proposed.

In 1971, Kimura and Kitao examined the pathogen obtained from diseased young yellow tails cultured at the farms of Miyazaki and Ehime pref., and determined that the pathogenic agent belonged to the genus Corynebacterium.

In 1972, Kusuda suggested that the disease was similar to 'white perch Pasteurellosis' which was reported by Snieszko et al. in 1964. He attempted to the serological tests for determing the serological types of P. seriola and P. piscicida, and obtained a result that these species were the same.

In 1973, the same sympton and pathological features were observed on red sea bream and other fishies in the farming field.

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CHARACTERISTICS OF *PASTEURELLA PISCICIDA* ISOLATED FROM HAMACHI INFECTED BACTERIAL TUBERCULOIDOSIS IN JAPAN

According to Kimura et al. (1971) and Kusuda (1972), the bacterium is slender, Gram's stain negative, straight or slightly curved rod shape measuring 0.6 micron wide and 1.6 micron long. Cells do not form spores and are non-motile in agar media. Cells exhibit pleomorphism and have metachromatic granulus at the both ends of the cells. Capsules are absent. Typical colonies of P. piscicida grown on brain heart infusion agar are the droplent-like in shape and colorless. The bacterium is a facultative anaerobe. Growth occured over the temperature range of 10°C to 30°C, and its optimum is between 23°C and 27°C. Growth of the bacterium is inhibited by 1.0–3.0% NaCl concentration. Its optimum pH is 6.5–7.5.

Nitrates are not reduced and neither indole nor hydrogen sulfide is produced. The oxydase test, methyl red test and catelase test are all positive. Gelatin is not liquefied. The organism attacks some carbohydrates, and produced acid from glucose, mannose, galactose and fractose, but othr sugars are not fermented.

The bacterium is selected to 3 serotypes, but unknown antigenicall composition of these types.

PATHOLOGY

The gross pathological changes in hamachi infected with *P. piscicida* are usually very limited. Frequently, there are little or no gross symptoms observable at the time of death. Slightly dropsial change and darkening of the color are only observed.

Histopathological changes, however, are very clearly and variable formed. The histopathogical reports of becterial tuberculoidosis are mainly investigated by Kubota, Kimura and Egusa.

Tubercle-like granules of kidney, spleen and other organs are common characteristics. And as one of the earliest pathological changes to be observed in fish suffering from this disease is a obvious edema of kidney and spleen. These edematous organs are discolored and have numerous tubercles.

The tubercles are constituted of many bacterial colonies, epithelioid cells, fibroblastes and other kinds of cells. These cells are surrounded by the bacterial colonies perfectly or not, and most often these are degenerated. The tubercles are round and measuring about 1–2 mm in diameter.

In acute infection cases, serious death without any macroscopic evidences can be observed.

The bacterium is found widspread throughout the fish, especially kidney, spleen, heart and other organs having well developed blood vessels.

There is little information about the changes of the blood characteristics of the disease. According to Kusuda, the hematological changes are obtained from youg hamachi in the experimental scales, and hemoglobin concentration of the infected fish is 5.95 g/dl, red cell count is 219 × 10000 cells/cubic mm, hematcrit value is 31.4% and serum protein concentration is 2.8–7.4 g/dl at the death time. And these values are obviously lower than the normal case. It is apparent that anaemia has occured in this case.

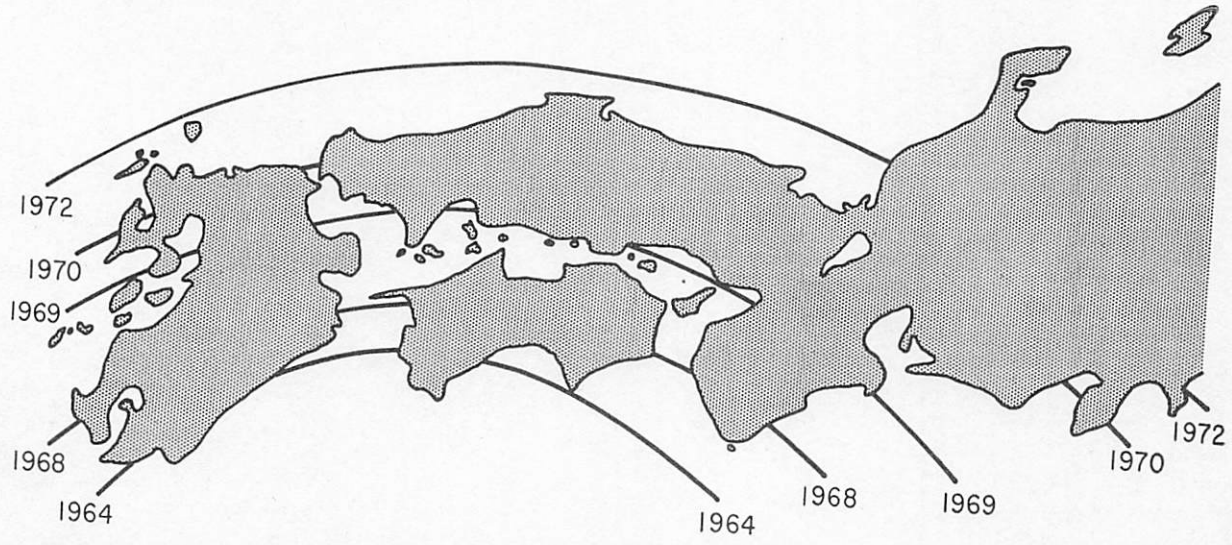

Figure 2. Dissemination of bacterial tuberculoidosis of culture yellow tail in Japan.
In 1964, this disease was recognized at the fish-farms in Miyazaki and Kochi prefectural, and during the period 1965–1972, it was widspreaded all south-western Japanese coasts.

ECOLOGY AND EPIZOOLOGY

In 1964, this disease was recognized at the hamachi culturing farms in Miyazaki and Kochi prefectural. Over the period 1964 through 1966, it was only one of the local disease, and very rarely it brought mass mortality.

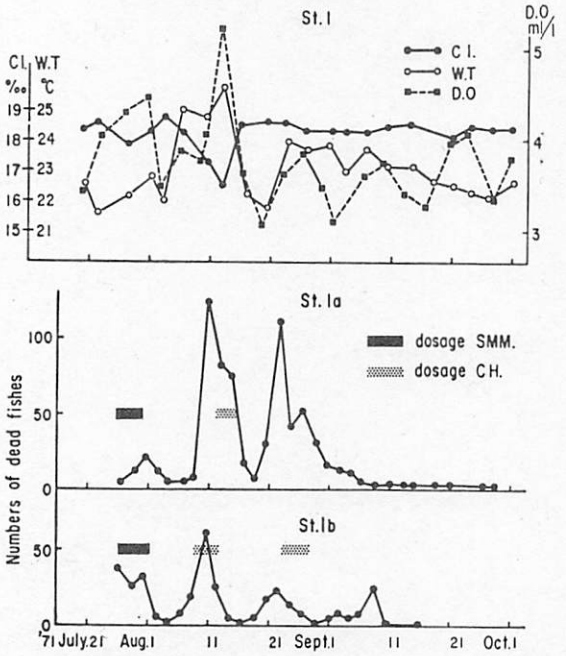

Figure 3. The relationship of the environment facters and daily changes of the numbers of dead fishes. This data was obtained at the fish-culturing farm of Numazu city, in 1971.

In 1968, this disease was disseminated to the southwest area in Japan, and serious mortality of young hamachi resulted. At that time, it occured once a year in early summer and was mainly occured among the young fishes about 10–15 cm in length. During the period 1969–1972, this disease was widspred along all Japanese coasts, and occured two or three times a year. (Figure 2) As shown by the investigations of the prefectural fishery experimental stations the most important factors concerning the disease is the water temperature and the salinity.

Our investigations from 1969 to 1972 suggested that the disease outbreak was related to the lower temperature in the lower salinity.

Usually, these environmental conditions are observed twice a year at the early summer and autumn. When the salinity of water becomes below 30–33‰ during the period of 'tsuyu' which is meant the rainy season in early summer in Japan and water temperature increases to 23–25°C, the disease occures. And at the autumn, after the rainy days the water temperature is down to 25–26°C, it must be careful for the prevalence. (Figure 3)

This disease is disseminated by the transportation of the juveniles is called 'mojako' in Japanese, and the disease is distributed in all culturing areas. Sometimes, some natural organisms living around the farming cages bring the disease to others places. The disease produced serious mortality to hamachi, and is increasing year by year. In 1972, the commercial

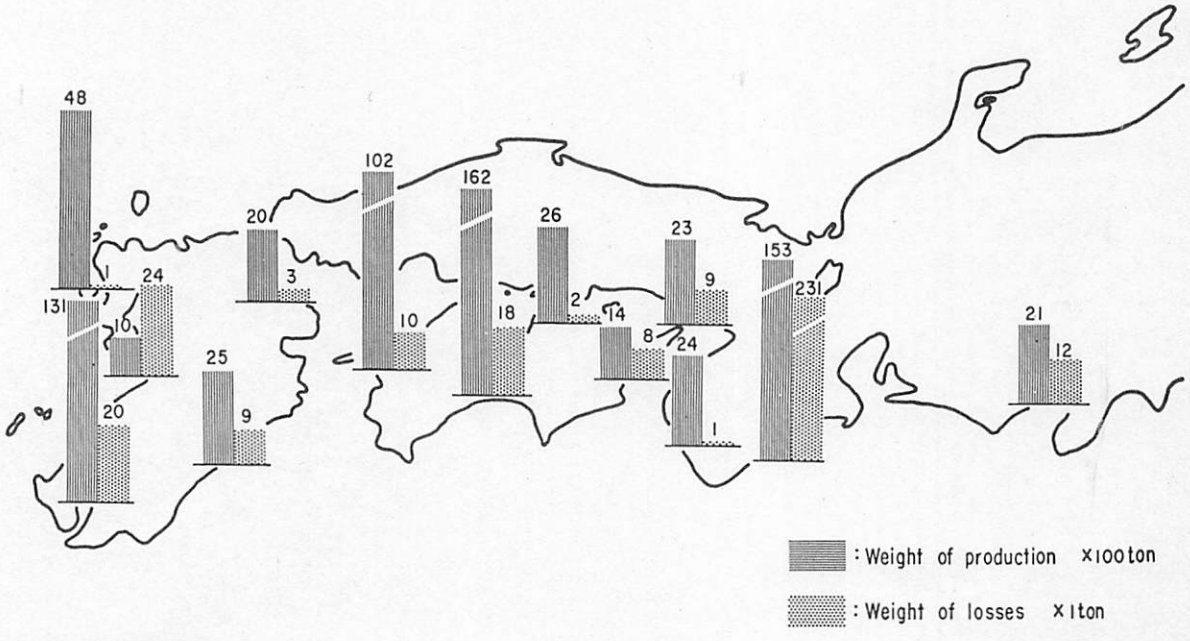

Figure 4. The "hamachi" production and the weight of losses by the disease in 1972.

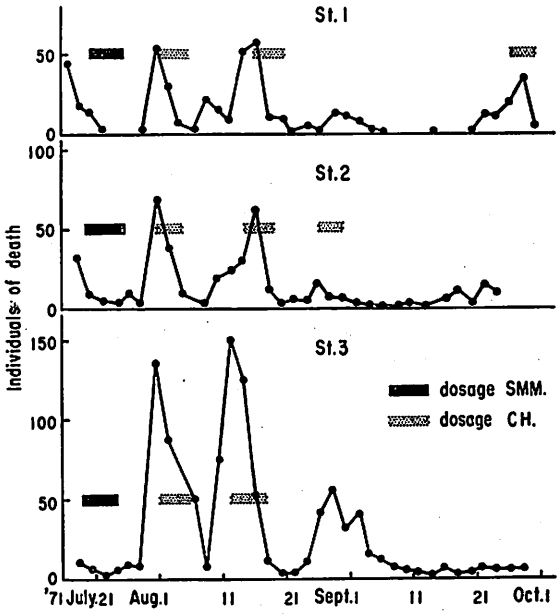

Figure 5. The effect of dosage of the drugs.
CH: Chloramphenicol, 20mg/1kg Body Weight/a day, mixing the drug with Oregon moist pelets.
SMM: Sulfamonomethoxine, 200–400mg/1kg Body Weight a day, Added to the food.

damage reached about 360 metric tons in weight, which is equivalent to 0.5–1.0% of the total product. (Figure 4)

PREVENTION AND THERAPY

The chemotherapy to prevent the disease had been studied. At present, sulphonamides, nitrofurazones and antibiotics are effective for the prevention. For therapeutic purposes, the sulposes, the sulphonamides are incorporated in Oregon-type pellets as 200–400 mg/kg in weight of fish a day for 6 days or more. Antibiotics, especially chloramphenicol is one of the most effective chemicals and widely used. It is mixed with the food about 20–40mg/kg/days or more. It is difficult problems that P. piscicida strains become resistant to several chemotherapeutic agents. (Figure 5)

DISEASES OF CULTURED KURUMA PRAWN *PENAEUS JAPONICUS* IN JAPAN

KUNIHIKO SHIGUENO[1]

ABSTRACT

In Japan, the pathological study of shrimp disease has been just started in conjunction with the occurrence of the diseases in the trials of intensive culture method where efforts were made to rear the shrimp at high stocking rates in tanks. Diseases also appeared in the course of breeding of larvae recently. As the study has only a few years' history, not much knowledge has been accumulated yet.

The diseases hitherto observed can roughly be classified as follows:

(1) black-gill disease due to *Fusarium solani*

(2) *Vibrio*-affected diseases at larval stages, characteristic of (i) occurrence of vermilion or red color along nervous system throughout body together with deformity and (ii) "white-turbid hepatopancreas" and

(3) *Vibrio*-affected diseases in the course of growing to marketable size.

Prior to entering an explanation of shrimp disease in Japan, a brief summary of the history and present status of shrimp cultivation may be necessary.

Since 1933 a number of biologists, most of whom joined the work initiated by the deceased Dr. M. Fujinaga have expedited their efforts for the development of techniques, which have developed step by step during the past thirty years excluding suspension during the World War II.

A number of trials and errors were repeated again and again for the betterment of technical processes. Among these, adoption of the brine shrimp, which appeared the market after the World War II, to the feeding of early post-larvae brought about an epoch-making event from which mortality due to improper feeding was minimized.

Secondly, in 1964 Dr. J. Kittaka developed a technically new approach to the breeding of larvae in large tanks; a massive propagation of plankton was held in the same tank where shrimp larvae in bulk had been bred, the growing of the mixed diatoms being induced by the application of chemical fertilizers into the tank water. From this approach was derived a newly standardized technique in the massproduction of larvae in the tank, the technique no longer depending on the skill of a specialized person.

Supported by these technical developments in the production of larvae, the pond culture of shrimp, on an industrial base was initiated by Dr. M. Fujinaga in 1963. The shrimp culture ponds, most widely developed in the Seto Inland Sea are established on deserted salt beds or on sandy beaches found along inner bays. A unit pond measures from 0.1 ha. to 10 ha. wide and the water sound 60 cm–180 cm for the sandy bottom. For convenient management, a unit pond of 3 ha. is common.

Surrounded by embankment, the pond takes in and discharges sea water through a water gate, taking advantage of tidal fluctuation. There are in Japan about twenty shrimp farms with ponds of more than one ha., most of which are located in the Seto Inland Sea with total area of ponds measuring about 150 ha. From these ponds nearly 400 tons of shrimp are produced in a year. The annual working schedule of shrimp culture is generally standardized: breeding of larvae in tanks in March, transferring of larvae to nursery ponds in April, releasing of the juveniles into growing ponds in June, and finally the harvesting that starts in September and lasts to mid-January of the following year, when the large shrimp have gradually thinned out.

The maximum product of shrimp usually reaches 300 g per square meter. The stocking rate is then limited to 15 individuals per square meter if the shrimp are expected to grow to 20 g: a high stocking rate of course lowers the weight of each individual shrimp. Shrimp production of 300 g per square meter is the maximum to be expected of one culture pond under the present conditions. Needless to say, this level of productivity could not be achieved in the ponds of old or in ponds today which are not provided

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with facilities to make proper change of water.

To produce 1kg of shrimp requires 7kg–10kg of fresh fish or shell-fish, 60%–70% of which should be shortnecked clam or blue mussel. The high demands for these bivalve shells today were preceded by the wide use of trash-fish and other small shrimp, which proved, however, nearly always to fail.

The main fishing ground of the short-necked clam can be said now to be restricted to the Suonada and Ariake Seas, and the other regions which used to grow them have been surrendered to expand industrial and agricultural reclamations along with water pollution. The cost of the short-necked clam has been rising like any other commodity, although fishing for clam still on a low technical standard, depends on manual labor.

In the early 1960's, when shrimp pond culture came into commercial operation and clams were fished as much as desired, the clam with its shell cost 2 U.S. cents a kilo delivered at the shrimp farm, but the cost rose to 5 U.S. cents in 1972.

In the working schedule of the shrimp culture as practiced in the Seto Inland Sea, the operation is limited to one cycle in a given year, taking advantage of summer weather for the growing. This shrimp naturaly do not grow in the winter season, so they are harvested from fall to early winter and shipped to the market during a more or less limited season. The products shipped from all farms, must compete with each other in the market. During the winter and into the spring months, the market quotations become higher due to the lowered supply. Increasing supply to meet increased demand is ruled out in terms of timing of shipment in case of shrimp pond culture as practiced in Japan.

The productive operation of the pond, once a year from April to December, is followed by a period of three months which are spent merely cleaning the ponds. Since the cleaning requires workers' hands, the productive potential of the pond is by no means economical in terms of labor, space, and timing.

Such the way of shrimp cultivation in ponds still prevalent in this country should be called an extensive or crude method so to speak. The relatively low populations of shrimp under such methods, largely depending on natural foods in the ponds, have never been affected by any serious disease. Or it may be better to say that any affected shrimp occasionally found lying at the pond-side were ignored or looked over as they were trivial in number. With the intention of developing improved techniques of shrimp culture by solving the problems incurred in past procedures, investigations have been going on since 1967 at Kagoshima Fisheries Research Station.

Firstly, studies have been directed to the preparation of formulated dry feeds and the operation of large concrete tanks established on land in which intensive culture of shrimp is conducted by feeding them compounded food materials.

This idea is based on the fact that the contemporary shrimp ponds established as they now are, immediately on the shore line of the sea, are hardly able to sustain thickly populated shrimp for rapid growth in stable conditions with a high survival rate. The shrimp ponds presently constructed on the sea shore appear as if they are functioning as a sedimention reservoir for organic substances, the general environment being diverted far from the habitats of marine animals which spend most of their time embedded in the bottom sand. These problems have to be approached from the angles which are encountered in the culture ponds of today. As the basic primary concept, the maintenance of clean water comes to mind. Substantial supply of clear sea water by pumping, and the maintenance of aerobic conditions in the bottom sand layer by the passage of water, were the subjects conceived in this idea. It was felt that under such an environment and through the feeding of formula feeds, the cultivation of shrimp in confined water could be highly advanced, technically and economically.

These basic ideas have been implemented in experiments in the past few years, and in practice, the ideas are now being worked out at some pilot farms operating on a commercial scale. Fortunately, after repeated trials and errors, a little more than one hundred tons of shrimp are expected to be produced by this newly developed techniques this year in four pilot farms.

However, since the beginning of these new trials we had to struggle with some newly found shrimp diseases which we did not ever notice in the traditional crude way of cultivation in the past.

Establishing measures to prevent and cure such shrimp disease under thickly populated rearing conditions have now become an important problem as well as to provide a favorable environment and fully nutritious formula feed.

Accordingly, the pathological study of such shrimp diseases has been just started in conjunction with the occurrence of the diseases in the growing tanks. Therefore the study has only a few years' history and not much knowledge has been accumulated. The diseases hitherto observed can roughly be classified into two cases due to fungi and gram negative bacilli.

(1) The disease of black-gill, caused by a form of imperfect fungi that is a relative of saprolegnia, is often found on the shrimp cultured in rearing tanks. Details of the infection were reported by prof. Shuzo

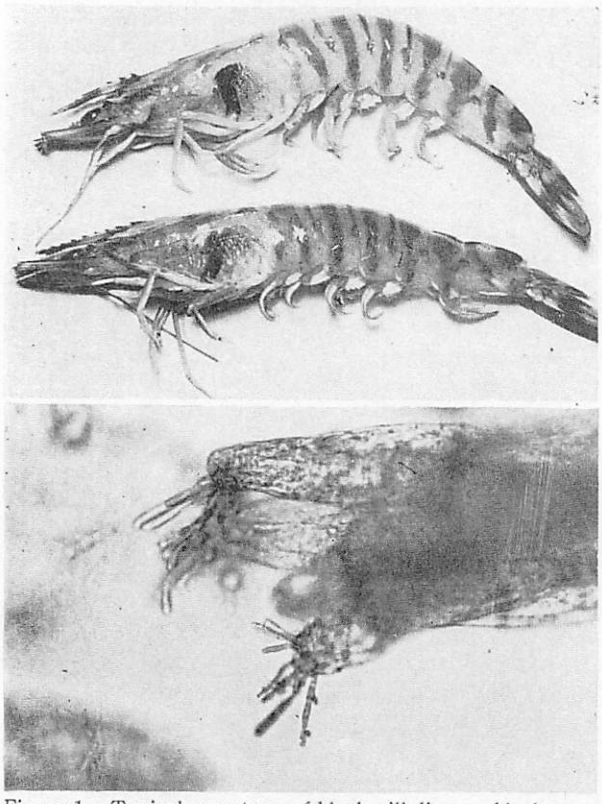

Figure 1. Typical symptoms of black-gill disease: black spots on the gills and the hyphae penetrating gill-lamellae.

Table 1. Examination of five shrimp suspected of black-gill disease showing occurrence of fungi *Fusarium solani* on the organs.

Sample	Antenna	Walking leg	Swimming leg	Telson	Eye ball	Gill	Stomach	Liver	Heart	Intestine	Muscle
1	−	−	##	−	+	##	#	##	#	#	+
2	−	−	−	−	−	##	−	−	−	−	−
3	−	−	−	−	−	##	#	−	−	##	−
4	##	−	−	−	−	##	#	−	−	−	−
5	#	−	−	−	−	##	−	−	−	−	−

abundant, # moderate, + positive, − negative.

Egusa (of Tokyo Univ.) and Tadao Ueda (of Kagoshima Fisheries Research Station).[1] Yasuo Fukuyo and S. Egusa identified it as *Fusarium solani* in 1974.

In shrimp infected by this disease the gills are stained by black spots. Severely infected gills collapse, and the gill-lamellae inside are lost by the penetration of the hyphae. The fungus isolated from the gill lesions is easily propagated in the culture medium of mycosel agar containing 1% NaCl. The conidia in suspension thus derived were inoculated intramuscularly into healthy shrimp which developed

Table 2. Results of checking of fungi and bacteria on 48 captured shrimp.

No.	Body Weight (g)	Amount of fluid tested (ml)	Fungi	Bacteria
1	29.0	0.30	−	+
2	23.6	0.50	−	−
3	20.5	0.35	−	+
4	23.6	0.30	−	−
5	30.4	0.40	−	−
6	26.5	0.30	−	−
7	32.0	0.30	−	−
8	15.0	0.20	−	−
9	26.4	0.30	−	−
10	16.5	0.25	−	+
11	24.0	0.40	−	+
12	19.1	0.15	−	−
13	29.0	0.30	−	−
14	22.1	0.40	−	−
15	36.6	0.20	−	+
16	34.4	0.40	−	−
17	34.2	0.30	−	+
18	25.0	0.30	−	−
19	26.0	0.25	−	+
20	31.1	0.50	−	−
21	35.6	0.20	−	+
22	30.5	0.20	−	−
23	28.6	0.30	−	−
24	29.6	0.15	−	−
25	29.8	0.30	−	+
26	27.1	0.60	−	−
27	27.1	0.25	−	−
28	43.4	0.50	−	−
29	36.6	0.35	−	−
30	30.3	0.40	−	−
31	27.0	0.10	−	−
32	22.0	0.20	−	+
33	22.5	0.25	−	−
34	24.1	0.25	−	−
35	35.6	0.50	−	−
36	23.2	0.20	−	−
37	20.2	0.30	−	−
38	21.6	0.50	−	−
39	19.1	0.10	−	−
40	27.5	0.40	−	−
41	24.9	0.30	−	−
42	23.2	0.10	−	−
43	14.6	0.15	−	+
44	33.5	0.10	−	−
45	31.7	0.10	−	−
46	35.4	0.50	−	−
47	17.8	0.10	−	−
48	32.6	0.15	−	+

+ for positive, − for negative

the disease. Some died a few days later, and all the shrimp treated died within two weeks. Examination of the gills of the dead shrimp confirmed the penetration of the hyphae into the lamellae surrounded by dark brownish pigment. Fig. 1 presents the typical symptom of black spots on the gills and the hyphae penetrating gill-lamellae.

As to the organs in the body of the shrimp, the fungus is found to attack the gills more heavily than other organs (Table 1), as confirmed in the examination of five shrimp 8.8g–14.8g in weight, which

were suspected of having contracted the disease.

The blood fluid of 50 shrimp showing the signs of black-gill disease, when cultured in the mycosel medium, all proved the existence of *Fisarium solani*. The same blood fluid of the 50 cultured shrimp, first treated in a clinical thioglycolate medium at 30°C two days, were plate-cultured in nutrient agar medium, and 43 individuals were found to carry unknown bacteria. A similar test was also made on the blood of 48 shrimp captured in the sea Table 2, and it was disclosed that 12 individuals carried a few unknown bacteria but none showed fungus infections. These findings are believed to suggest that bacteria or mold fungi entering the body of shrimp are easily dispersed to various parts of body through its open vascular system.

(2) Several diseases due to pathogenic bacteria are reported in the breeding of larvae and in the course of growing them to marketable size. The identification of each pathogen, measures of prevention, and cures are respectively being undertaken and explored in some laboratories. Hence, the following description is limited to a brief introduction of the typical symptoms of each disease.

Since 1971 heavy losses of shrimp larvae at the stage of mysis and post-larvae in the breeding tanks are often reported by many farms. There seems to be at least two different types of symptoms, both of wich are considered due to infectious *Vibrio spp.*

The one observed in Kagoshima is preceded by incomplete moltings in the mysis and post-larva stage. The larvae lose some parts of their appendages, and eventually become handicapped and almost annihilated in the following one or two days.

In the initial stage of this disease, some parts of the larvae in the tank are observed to have yellowish vermilion and red color along the nervous system throughout the body.

In 1974 at Kagoshima, efforts were made to overcome this disease presuming that there may have been some preceding occasion which weakened the vitality of the larvae and permitted the intrusion of the pathogenic bacteria. In this respect, there are some inherent defects in the propagation of mixed diatom by the application of chemical fertilizers into the breeding tanks. Density and the species composition can never be controlled. Accordingly, it often happens that some indigestible species of diatom such as *Navicula spp.* or *Coscinodiscus spp.* predominate the flora in the tanks, where growth and metamorphosis of the larvae are inevitably delayed, causing the weakened vitality of the larvae.

In this regard, Isamu Setoguchi et al. (1974) have successfully undertaken a series of experimental breedings by means of a diatom-pure-culture combined with artificially compounded feed. The composition of the ingredients of the test diet H–105 is as follows.

Ingredient	Part in weight
Squid meal (*Ommastrephes solani pacificus*)	47
Shrimp meal	15
Fish protein concentrate	5
Skipjack testis meal	10
Beer yeast	5
Wheat gluten	3
Activated sludge	5
Activated starch	5
Vitamin mix.	2
Mineral mix.	13.7
Total	110.7

As regards the species for pure-culture, the single-celled *Chaetoceras sp.* had previously been isolated from the sea water. This species, able to stand the comparatively high temperature of the water and being easily digestible by the larvae, is quite fitted to the breeding of shrimp larvae in tanks. One of the reasons why the diatom-pure-culture method had been abandoned (since 1962 in Japan) was due to the fact that *Skeletonema costatum* could not be cultured in a stable condition in water above 25°C. Also the sterilization of large quantities of tank water in a short time was thought unrealistic. This has also been overcome by means of chemical sterilization with a sodium hypochlorite solution (about 10% chlorine). Followed by neutralization with sodium thiosulphate twelve hours later, the tank water becomes ready for chemical fertilization of the isolated *Chaetoceras sp.*

Consequently, the limiting factors that in previous years hampered the scheduled mass-propagation of the selected diatom on an industrial scale have already been cleared away. The operational procedure has been realistically simplified and standardized. It has been proved that by adopting this method no more bacterial disease in the course of breeding scarcely occurs, and the mortality results almost in nil.

Another disease with different signs often seen in Yamaguchi area can be explained as follows. Like its provisional name "white-turbid hepatopancreas",[4] the midgut gland of the larvae becomes white-turbid in general. As the disease progresses, the white turbidity becomes more apparent and well-defined.

The heavily infected post-larvae, mostly 6mm–9mm in length, can easily be distinguished, as they float inactively on the surface of the water and exhibit white intestinal lines. The cells in the infected liver are largely corroded by gram-negative bacteria. It is also observed in rare cases that blotches of brownish black pigment emerge in the midgut. According to

Table 3. Lethal effects of intramuscular injection of bacteria on shrimp.

Stock No. of bacteria.	Part of body where bacteria were isolated.	Conc. of bacteria, 10^5/ml Time elapsed and number of shrimp killed.				Conc. of bacteria, $10^5 \times \frac{1}{5}$/ml Time elapsed and number of shrimp killed.				
		6hr	8hr	11hr	22hr	6hr	9hr	11hr	22hr	46hr
1	Blood	0	0	1	0	0	0	0	0	0
2	〃	0	0	1	2	0	0	0	0	1
3	Hepatopancreas	5	—	—	—	4	1	—	—	—
4	Blood	0	0	0	2	0	0	0	0	0
5	〃	0	5	—	—	0	3	1	1	0
6	〃	0	2	3	—	0	4	0	1	—
7	〃	0	0	2	2	0	0	2	1	1
8	Hepatopancreas	0	0	2	3	0	0	0	4	1
9	〃	0	0	1	1	0	2	1	0	1
10	〃	0	0	0	5	0	1	0	1	0
Control	Injection of physiological salt solution	0	0	0	0	0	0	0	0	0

a report from the Yamaguchi Inland-Sea Fisheries Research Station, high mortality (60%–100%) occurred in 9 out of 31 breeding operations performed at the Yamaguchi Marine Seedling Center in 1973.

The infections of bacterial disease in the course of growing to marketable size are briefly introduced as follows: In 1971, many shrimp died in the experimental rearing tank at the Research Station during August to October. In nearly the same period a number of shrimp died in similar condition at a shrimp farm located 30km north of the Research Station. These dead shrimp in both places initially showed weakened activity on the surface of the bed. As the disease progressed the basal part of the antenna, the basal part of the oviduct and seminal-duct (posterior to heart), the hepatic carina on the carapace, and the posterior and lateral edges of the abdominal shells all became blackend or whitened (K. SHIGUENO, 1975). On some individuals, the color changes spread to the second antenna and uropods.

Picking up those shrimp that had appeared on the bed, T. Ueda attempted the temporary breeding of bacteria in ten stocks, (Nos. 1–10) from the samples collected in the blood and liver. Each of the ten stocks was then inoculated intramuscularly to shrimp which appeared to be in good health, and the dying of these animals was traced. Each of (SHIGUENO, 1975) the separated bacteria was cultivated in a nutrient agar medium (1% salt added) for twenty hours, and were then placed in a physiological salt solution of the same concentration (10^5/ml.) or turbidity and checked by a spectrophotometer. An inoculation of 0.2 ml. of each of the stocks was injected into five shrimp weighing 6g in the third abdominal segment. The results in Table 3 showed that stocks Nos. 3, 5 and 6 killed all five shrimp within ten hours. The same injections diluted with 20% of the original stocks also proved the high toxicity of Nos. 3, 5 and 6 killing all the shrimp within twenty-two hours.

Stock No. 3 is derived from the hepatopancreas of the shrimp reared in the experimental tank at the Kagoshima Fisheries Research Station; No. 5 from the basal part of the second maxilliped of the shrimp of the same origin as No. 3; and No. 6 from the blood of the shrimp from the Pilot Farm. The shrimp tested as control specimens were not affected. The bacterial examination as conducted above proved that infection by pathogenic bacteria was responsible for the mass mortality of the shrimp.

T. Ueda, after repetition of oral application of varied concentrations of sulfisozole, nifurstyreic acid, and chloramphenicol compounded in formula feed, found that these drugs were effective in saving the pathogen-inoculated shrimp. It was also made clear that the drugs absorbed in the intestine concentrated mostly in the hepatopancreas, followed by the gills and muscles. In his study T. Ueda identified all four bacteria as *Vibrio* or related forms. He also found through a serum coagulation test on rabbits that all four bacteria were specifically different.

A kind of bacteria, not yet identified, is considered responsible for "gill disease." In the course of growing at high stocking density in a culture tank, the shrimp happened to encounter this infectious gill disease, especially in the spring season. In the initial stage of this disease, the color of the gills turns dull orange-yellow or light brown. As it becomes serious, the color turns darker until it is finally black (SHIGU-

ENO, 1975). As a countermeasure to this disease, bathing in furazolidone at a concentration of 2 ppm.–3 ppm. is recommended. The bathing is undertaken for two to four consecutive nights, depending on the condition. The loss of shrimp by this disease is usually not as serious as the diseases previously mentioned.

These are the outline of shrimp disease hitherto obseved in Japan; no microsporid disease ever reported.

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