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Health management in aquaculture

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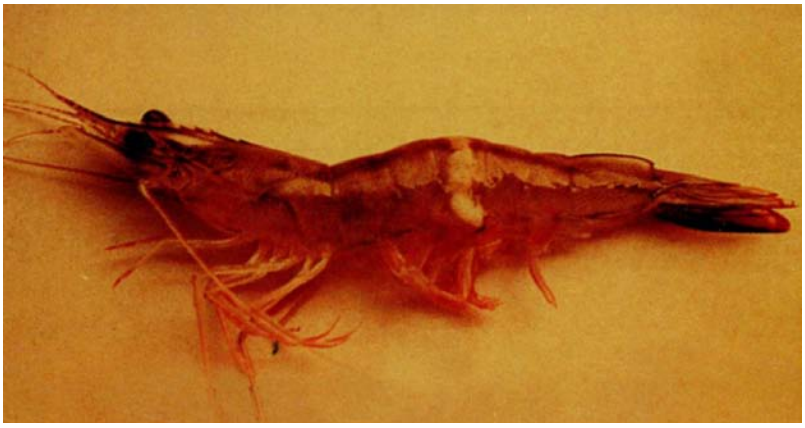
HEALTH MANAGEMENT IN AQUACULTURE



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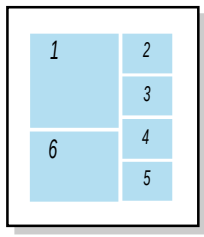


1 fish kill in a milkfish pen in Pangasinan, northern Philippines (PHOTO BY A DE LAVEGA);

2 visual inspection of grouper fry in the SEAFDEC hatchery (PHOTO BY R BUENDIA);

3 examination of a slide under the microscope (PHOTO BY R BUENDIA);

4 the molecular biology laboratory at SEAFDEC (PHOTO BY R BUENDIA);

5 monitoring of water parameters (PHOTO BY R BUENDIA);

6 shrimp with microsporeans on the abdominal muscle (PHOTO BY R DUREMDEZ)

Foreword

The need for specific educational materials on aquaculture relevant to the tropics has, for several years, been raised in many meetings attended by representatives of state colleges/universities, government extension offices, and R&D institutions. The need for textbooks became more urgent when a few of the colleges/universities started offering fisheries degree programs through the distance education mode.

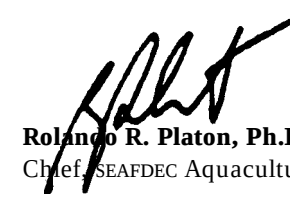
SEAFDEC/AQD's textbook writing project rapidly came into fruition upon the urging of the Iloilo State College of Fisheries (ISCOF). AQD has been reputed to house the most number of experts in various fields of aquaculture, and we are only too glad to share our expertise and results of decades of research and development.

This textbook on Health Management in Aquaculture is the beginning. It contains the most up-to-date knowledge of fish and crustacean diseases, the causative organisms, and the measures for disease prevention and control in tropical aquaculture.

Although the primary target reader is the student, there are also other stakeholders in the aquaculture industry who can use the book for quick reference - the fish farmers, farm workers and technicians, fishers and women in fishing communities.

To our readers, we urge that you always bear in mind that aquaculture does not exist in isolation. Aquaculture affects, and, in turn, is affected by its surrounding environment. The responsibility of aquaculture is to increase food supply without damaging the natural support ecosystems. The issue of fish health in particular is illustrative of this paradigm. Widespread disease problems can occur only when the culture environment deteriorates to the point that it favors the growth of disease-causing organism more than the welfare of cultured species. Severe economic loss is just one of the consequences.

We hope that this book would contribute immensely to the study of fish health in aquaculture.



Rolando R. Platon, Ph.D
Chief, SEAFDEC Aquaculture Department
June 2001

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Preface

The preparation of written lectures in training courses at SEAFDEC/AQD was initiated in 1982. This was spearheaded by Dr. Chua Thia Eng upon implementation of the Training Course for Senior Aquaculturists in Asia and the Pacific, a collaborative venture among SEAFDEC/AQD, the Network of Aquaculture Centres in Asia (NACA) and the University of the Philippines in the Visayas (UPV). Since then, the Fish Disease course of NACA set the format for the Fish Health Management training courses at SEAFDEC/AQD that began in 1987. The compiled lecture notes became a reference for SEAFDEC trainees and the students of local fisheries schools. Thus, in response to the Iloilo State College of Fisheries (ISCOF) urgent need for a textbook, the fish health reseachers at SEAFDEC endeavored to modify their written lectures to textbook standards. Despite existing lecture notes, it took more than a year to complete.

This is the first textbook on diseases of cultured warmwater fish and shrimps in the Philippines. Eleven chapters cover essential information on the basic principles of disease causation, major diseases of cultured fish and crustaceans, particularly shrimps, and methods of prevention and control. Emphasis is made on major diseases that occur in the Philippines and other countries in the Asian region. Included also are topics on harmful algae, immunology and molecular biological diagnostic techniques. Each chapter begins with a brief preview of its contents, followed by an in-depth discussion that culminates in a summary. The basic concepts presented were derived from textbooks on microbiology and fish health published by authors outside of the Philippines while the scientific information put forth were drawn from the research and diagnostic findings of the authors, colleagues at SEAFDEC and other institutions in the Philippines and in other countries. The figures and photos included were selected from the authors' collection of disease cases or shared by colleagues in this field, duly acknowledged at the end of the book. Specific citations were omitted in the text as students are enjoined to refer to references used for each chapter. To make the concepts more understandable, a glossary is included. An index is also part of this textbook for quick location of desired subtopics. Finally, although laboratory protocols are an essential aspect of fish disease diagnosis, it is best dealt with separately. Interested readers are referred to published manuals.

Though the authors have strived to make this book learner-friendly, we realize that a first edition leaves much room for improvement. We, therefore, encourage colleagues to suggest ways of enchancing the future second edition responsive to the needs not only of students but those of aquaculturists as well. It is hoped that this textbook will answer questions on fish health in scientific ways and enhance aquaculturists' ability to prevent diseases and deal with outbreaks effectively.

Finally, this textbook has become a reality with the support of SEAFDEC/AQD and its Chief, Dr. Rolando Platon; ISCOF President Dr. Elpidio Locsin Jr; Rene Agbayani, Pastor Torres Jr, Mila Castaños, Nicanor Primavera Jr, Edgardo Ledesma and Dr. Evelyn Grace de Jesus.

GD Lio-Po
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CHAPTER ONE

Disease development

Celia R. Lavilla

The need for more and more food fish to feed a growing population gave rise to aquaculture. Today, aquaculture yields have increased, with more harvests coming from farms in Asia.

Aquaculture has three phases – the (1) hatchery, (2) nursery and (3) grow-out phases. Most hatchery operators use tanks to hold the young organisms that they grow. In the nursery and grow-out phases, tanks, ponds, and floating cages hold the farmed animals until harvest time.

In aquaculture, any one of three production systems may be used in growing the chosen species. These are the extensive, semi-intensive, or intensive production system. Choice of a system depends on the desired density of animals to be farmed in a given area. In the intensive system, fish are farmed in high stocking density. High stocking density results in exposure of the animals to stress that often leads to disease. Disease outbreaks, in turn, cause production losses due to lower harvests or aquatic products of poor quality.

Disease is defined as any abnormality in structure or function displayed by living organisms through a specific or non-specific sign (symptom). Infectious organisms, wrong management practices and environmental problems can cause disease in farmed aquatic animals. Tissue or organ damage, reduced growth rate, or death may indicate disease in fish. The consequence of disease includes rejection of aquaculture products and the loss of productivity. Persistent disease occurrence might cause the collapse of aquaculture ventures and threaten the sustainability of the industry as a whole.

Because of their harmful effects, disease and environmental problems have gained worldwide attention. Although economic losses due to diseases in aquaculture are difficult to measure, data gathered from the export of various aquatic commodities may serve as indicators for losses or gains in production. For example, China's export figure for farmed shrimp in 1992 was 140,000 metric tons. In 1993, shrimp export went down to only 30,000 metric tons. Viral disease caused the 79% reduction. Translated to export earnings, the country lost about a billion dollars from shrimp alone. An ADB/NACA (1991) estimate of losses in aquaculture due to disease was about US$1.36 billion in 15 Asian countries in 1990. The situation is even worse at present with viral disease plaguing shrimp culture facilities worldwide.

HOW DISEASE DEVELOPS

The development of disease in a particular aquaculture system involves several factors: the farmed fish (host), the disease-causing organisms (pathogens) and the surroundings (environment). A complex interaction exists among these three factors as represented in the diagram of three overlapping circles (Fig. 1-1). For a disease situation to exist, there should be a potential pathogen, a susceptible host, and environmental conditions that bring about either increased virulence of the pathogen, or decreased resistance of the host.

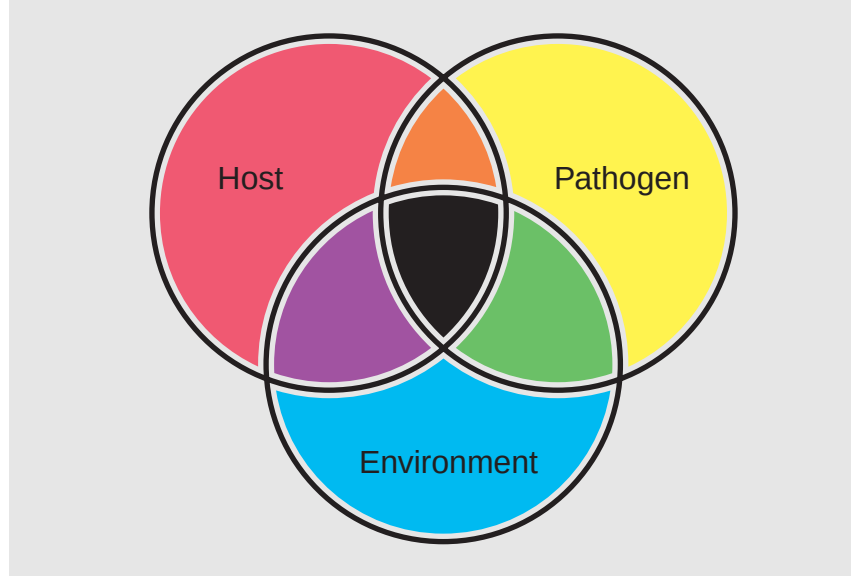

Figure 1-1. Snieszko's (1974) diagram of the effects of environmental stress on outbreaks of infectious diseases of fish. Factors that are related to the disease organism, host, and environment must be present for the disease state to occur. In the diagram, the blackened triangular area resulting from the overlap of three circles signifies this

Hosts

A host (fish or any farmed aquatic animal) can be either resistant or susceptible to a given disease. Resistance or susceptibility of the host depends on (a) age or size of the host organism, (b) species, (c) defense mechanisms employed and (d) the health of the fish, including its nutritional state.

Pathogens

Agents of diseases or pathogens can be classified into physical, chemical, and biological agents. Extreme temperature changes and radiation, such as ultraviolet rays from the sun, are examples of physical agents. Chemical agents can cause illness in aquatic organisms in a variety of ways. Environmental contaminants, toxins, nutritional imbalances and drug and chemical overdose are classified as chemical agents of disease.

Biological agents may initiate disease. They are the primary focus of attention when dealing with infectious diseases. These agents include viruses, bacteria, fungi and parasites. They are often called infectious disease agents. They can be present in the water or in sediment as part of the normal flora. Their presence and number are largely influenced by environmental factors like tempera-

ture, dissolved gases, pH, and availability of food. The two major characteristics of an infectious agent are (a) capability for direct transmission and (b) ability to multiply in the host tissue. The mode of their transmission is either (a) vertical or (b) horizontal. In vertical transmission, infectious agents transfer from parent to offspring. The female or male broodstock may be carriers of diseases, and transfer them to their offspring through the egg or sperm. In horizontal transmission, infectious agents come in contact with the hosts through the water, the feeds or through carrier animals that are in the environment.

Environment

The environment of cultured fish is composed of the water and its holding system like tanks, ponds, cages, pens, etc. Stability of the environment, especially in the physico-chemical parameter of the water brought about by the fish culture activity itself or by natural causes, will determine the health of the fish. Fluctuations in temperature, pH, salinity or dissolved oxygen beyond the optimum range for the host may lead to stress and disease. The key to successful fish culture is to understand and manage the environment of the host organism. Understanding the role of the environment in affecting the nature and cause of disease is essential to the prevention and control of disease.

Human Factor

The human element is an important consideration in the farming of aquatic animals. Aquaculture technicians and other personnel should have adequate knowledge and understanding of the species they culture to assure the success of an aquaculture operation. Lack of experience or insufficient personnel may lead to costly mistakes and poor yield. Farming strategies should consider the crucial role of the people involved in every stage of production.

Stress in Disease Development

Handling, overcrowding, malnutrition, or poor environmental conditions are stressful to cultured fish. Stress is defined "as the sum of the physiological responses the fish makes to maintain or regain its normal balance." Response and adaptation to stress takes place in three phases (adapted from Roberts 1978):

a. In the *alarm stage*, the fish attempts to escape from the problem.

b. If escape is not possible, the fish's body attempts to react to the environmental change. This is the *adaptive stage*, where the fish tries to adjust to the change and reach a new equilibrium, both physiologically and behaviorally, to survive the new environmental conditions. Although fish may successfully adapt to new and changing conditions, its growth, reproductive capacity and disease immunity may not be maintained as in the previous level because, at this stage, the fish deals with the stress as a priority. It is in this stage that disease problems are more likely to occur. Extended exposure of fish to environmental deterioration disturbs its normal function and decreases its chances of survival

c. If the environmental change is so great that the fish cannot adapt to it, then the fish stress response finally reaches an *exhaustion stage* and the fish dies.

Abrupt change in salinity, pH or temperature, especially at levels beyond the animals' tolerable range, cause significant stress in fish and make them succumb to secondary infection due to opportunistic microorganisms. Exposure to stress may also weaken the defense mechanism of fish.

DISEASE DIAGNOSIS

Disease diagnosis involves recognizing the occurrence of an abnormality and identifying its cause. Diagnosis of fish disease is a relatively new service available only fairly recently and the range of laboratory procedures is still limited. The eventual findings are not always very definitive, though the elimination of certain possibilities may still prove useful. A meaningful diagnosis is most likely where the fish are under the closest supervision, and where the fish farmer is able to provide data on environmental parameters and management practices employed.

Signs of Diseases

A sick fish often exhibits some disease signs before it dies. The first indication may be reduced feeding. Abnormal changes in fish color and behavior are among the earliest signs seen in affected fish. The fish may stay away from the school, or swim at the surface or along the tank sides. The fish may also exhibit flashing, scraping on the bottom of projecting objects, darting, whirling or twisting, and final loss of equilibrium. In addition to these changes, body surface abnormalities and lesions may be observed externally or internally. Specific disease signs associated with various diseases are presented with the diseases covered in this book.

Diagnosis

Fish disease diagnosis follows a format similar to that applied to other animal species, with more importance given to water quality parameters because of their direct effect on fish. A good history of the disease should be supported by personal observations before performing postmortem examinations. Following a gross appraisal, the most usual routine procedures are parasitological examinations, bacteriology, and histopathology. The last two procedures may require laboratory support. The following lists of on-site and laboratory procedures to investigate disease outbreaks are recommended by Anderson and Barney (1991):

On-site investigation:

- Examine fresh materials from healthy, moribund, and dead fish;
- Collect fish tissue samples;
- Measure environmental conditions (temperature, oxygen, etc.);
- Investigate physical factors and rearing conditions; and
- Gather information on time-course of mortalities.

- Deliver suitable samples with accompanying information to the diagnostic laboratory as soon as possible.

Laboratory procedures:

- Presumptive identification of pathogens (viral, bacterial, fungal, parasitic, etc.);
- Positive identification and confirmation;
- Test for drug sensitivity and effectiveness; and
- Evaluation of recommendations and reports of additional analysis (histopathology, toxicology, etc.).

Correct diagnosis is essential in selecting the best management approach to correct the problem and the best possible treatment for the disease. It will be useful as a reference for installing future disease-preventive aquaculture procedures and practices. Because diagnostics require suitable laboratory facilities and trained personnel, final diagnosis of fish disease should be performed in accredited Fish Health Laboratories.

Surveillance and Monitoring

Hatchery or farm personnel can initiate disease diagnosis at the farm level. This is done through regular monitoring of cultured populations, feed, and environmental inputs to detect the presence of opportunistic pathogens. One must be familiar with the normal condition of the fish to be able to easily spot any changes or abnormalities. Without an adequate record of the normal health status of the fish, deviations from the normal cannot be assessed. Consistent record keeping is vital in monitoring the health status of any cultured population. The keeping of records is also necessary to enable farm personnel to identify environmental problems, learn from past mistakes, and to minimize production costs.

The need for regular monitoring is directly related to the intensification of the production system. For extensive systems without feed inputs, it may be enough to provide minimal monitoring. In hatchery operations, and when engaged in the semi-intensive or intensive systems, however, regular monitoring procedures should be applied. Some examples of needed monitoring procedures are periodic microscopic evaluation of gill tissues to assess the level of microbial gill fouling, daily microscopic examination of larvae from culture tanks, and evaluation of culture tank water algae and other feed inputs.

When a disease outbreak is encountered, data gathered on the pattern of losses, the species and sizes of fish affected, and the duration of the epizootic can provide a great deal of useful information. Sudden explosive die-off involving all fish present usually indicates the presence of an acute environmental problem. This may be manifested by lack of oxygen, the presence of a lethal chemical toxicant, or by lethal temperatures. Mortality that starts with the appearance of a few sick fish, unusual behavior, or loss of appetite can signal the onset of infectious disease. These diseases can be more or less acute or prolonged in duration and the mortality ranging from high percentage of fish dying on a daily basis to low percentage of mortality over several weeks (Fig. 1-2).

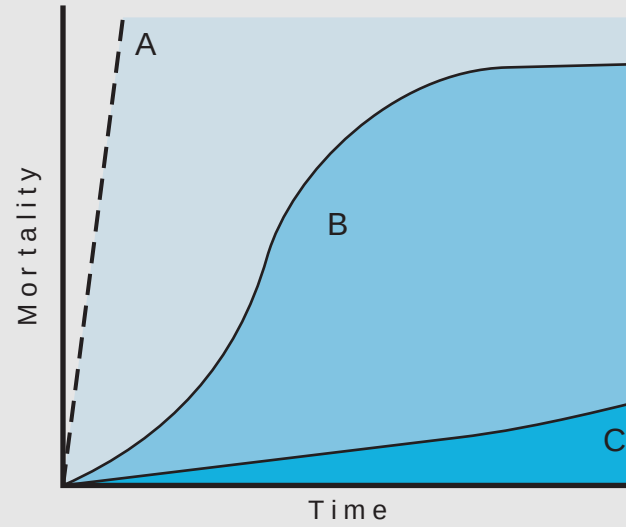

Figure 1-2. Hypothetical patterns of mortality of fish suffering from various kinds of diseases. (A) Acute environmental failure; (B) Acute infectious disease; (C) Chronic infectious disease

Ease in monitoring and collecting samples depends on the development stage and market value of the fish. The number of samples obtained from specific batches of fish is important. This is because adequate monitoring and diagnosis are impossible without sacrificing animals. Table 1-1 shows the recommended number of samples needed to represent a certain population of hatchery-reared shrimp larvae being monitored.

Larval population (Number of Larvae)			Sample size
1,000	to	199,000	20
200,000	to	399,000	40
400,000	to	599,000	60
600,000	to	799,000	80
800,000 up			100

Table 1-1. Recommended number of samples for different populations of larval shrimp being monitored.

However, if the prevalence of a certain disease without clinical manifestation needs to be assessed, then the number of animals to be examined should be based on assumptions that would yield statistically significant results. Table 1-2 gives the number of samples to be examined given an assumed prevalence of the disease.

Table 1-2. Recommended number of samples to test under various assumed prevalence of disease in the population

Prevalence of disease in the population (assumed)	Number of samples to test
1% or higher	300
2% or higher	160
5% or higher	60
10% or higher	30

SUMMARY

Disease occurrence is one of the biggest deterrents to sustainable production in aquaculture. It is therefore important to enhance awareness among various sectors of the importance of health management in the aquaculture industry. This can be done through education and information dissemination.

Students in fisheries and veterinary medicine need to have adequate background information on the aquatic animal disease and health management to understand the problems and needs of a fast-growing aquaculture industry. Recognizing disease signs early and using mortality pattern as a clue to the disease agent involved will not only make diagnosis easier, but it will also prevent massive losses by timely implementation of remedial measures.

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CHAPTER TWO

Viral diseases

Gilda D. Lio-Po

Outbreaks of viral disease in cultured fish and shrimps have been more frequently reported in the past two decades. Its significance cannot be ignored as their occurrences resulted in heavy mortalities. This chapter aims to provide basic information on viral disease problems involving economically important fishes, such as catfish (*Clarias* spp. and *Ictalurus* spp.) snakeheads (*Ophicephalus striatus*), carp (*Cyprinus* spp.), tilapia, milkfish (*Chanos chanos*), grouper (*Epinephelus* spp.), rabbitfish (*Siganus* spp.), sea bass (*Lates calcarifer*), mullet (*Mugil cephalus*) and penaeid shrimps.

CHARACTERISTICS OF VIRUS

Viruses are ultramicroscopic organisms with size ranges of 10 to 300 nanomicrons (nm). An electron microscope is required to visualize viruses. Because of their size, viruses are able to pass through filters of 0.5 micron pore size.

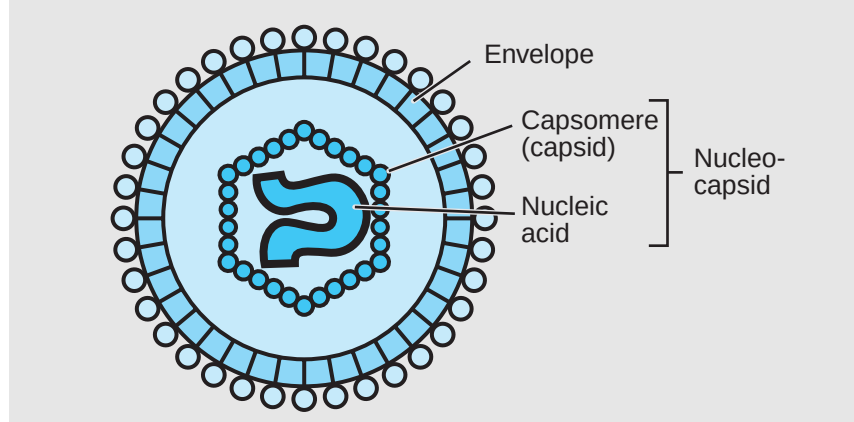

Figure 2-1. Structure of an enveloped virus

The basic structure of a virion consists of a capsid which encloses a nucleic acid genome (Fig. 2-1). The capsid is made up of identical protein subunits called capsomeres while the genome is either a ribonucleic acid (RNA) or a deoxyribonucleic acid (DNA). The combined viral components is called the nucleocapsid. This may have an envelop which is lipid in nature. Viruses that do not have an envelop are considered naked viruses.

Viruses have cubical/polyhedral, helical or complex morphology/symmetry. These microbes are obligately parasitic, multiplying only in its live host. In animal cells, the virus initially attaches on specific cell surface components called receptors. Subsequently, the whole virion penetrates the cell then makes use of the metabolic machinery and pathways of the living cell to make copies of its nucleic acid and synthesize protein subunits. Thereafter, these basic structural components are assembled to package the new viral particles. The new virions are usually released from the cell by lysis.

Cells infected by some virus develop inclusion/occlusion bodies within the infected cell as the replication cycle progresses. These are formed as a result of an accumulation of virions or viral components, although some inclusion bodies may not contain virions. These could be singular, multiple, intracytoplasmic or intranuclear and can be detected by histopathology using a compound microscope.

Aquatic viruses are transmitted from fish/shrimp to other fish/shrimp, from water to fish/shrimp or from reservoir to fish/shrimp by horizontal transmission. Disease transmission can also result from brooder to eggs/fry via vertical transmission. Known reservoirs of viral pathogens are farmed fish/crustacean, imported fish/crustacean, wild fish/crustacean, other aquatic animals/plants and survivors of viral epizooties.

Diagnosis of viral infections can be made by a combination of various methods such as signs of disease, detection of inclusion/occlusion bodies, electron microscopy (EM) or infection enhancement bioassay. Tissue culture techniques using established fish cell lines i.e. BB (Brown Bullhead), BF2 (Bluegill Fin), CCO (Channel Catfish Ovary), CFS (Catfish Spleen), CHSE (Chinook Salmon Embryo), EPC (Epithelioma Papulosum Cyprini), FHM (Fathead Minnow), GCK-84 (Grass Carp Kidney), GCG (Grass Carp Gonad) and GCF (Grass Carp Fin), RTG-2 (Raibow Trout Gonad), SBK-2 (Sea Bass Kidney), SHS (Snakehead Spleen), SSN-1 (Striped Snakehead Fry). To date, however, no continuous cell line from shrimp has been established.

Filtrates prepared from homogenized tissues of virus infected fish when inoculated onto monolayers of susceptible cells will result in lysis/destruction of the cells known as cytopathic effect (CPE) . Serial dilution of the filtrate will provide information on the estimated titer of the virus expressed as tissue culture infection dose (TCID). Alternatively, the focal site of CPE induced by each virion will cause the development of a clear zone called plaque. In such plaque assay, the number of plaques formed indicates the estimated viral particles in a given sample.

Other techniques based on the principle of serology are also applied in the diagnosis of viral infections. The more commonly used tests include neutralization index (NI) determination, Western Blot, Enzyme-linked Immunosorbent Assay (ELISA), Fluorescent Antibody Technique (FAT) and Indirect Fluorescent Antibody Technique (IFAT). Recent molecular biology techniques such as Polymerase Chain Reaction (PCR), Reverse Transciptase-Polymerase chain reaction (RT-PCR), DNA Probe have been developed and is currently widely applied for diagnosis of viral infections in shrimp.

In establishing the pathogenicity of a virus, provisions embodied in the River's Postulates are followed:

- The virus must be present in the host cells, blood, or body fluids showing specific lesions at the time of the disease.
- Filtrates of infectious material, blood or tissue, shown not to contain bacteria or other visible cultivable pathogen in inanimate media, must produce the disease or specific antibody in appropriate animals.
- Similar filtrates from animals or plants must transmit the disease.

It is only when these provisions are complied with that a virus can be considered pathogenic to fish or shrimp.

MAJOR VIRAL INFECTIONS IN FISH

Epizootic Ulcerative Syndrome (EUS)

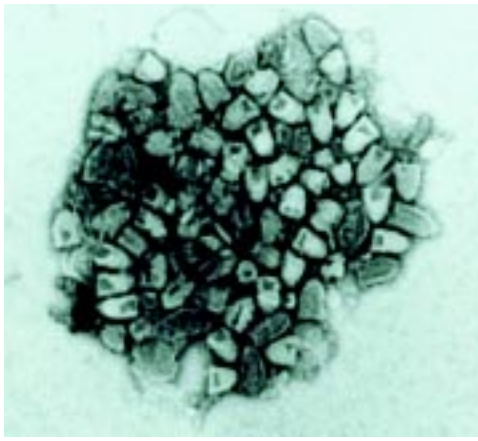

Figure 2-2. Electron micrograph of rhabdovirus isolated from EUS-affected snakehead *(Ophicephalus striatus)*

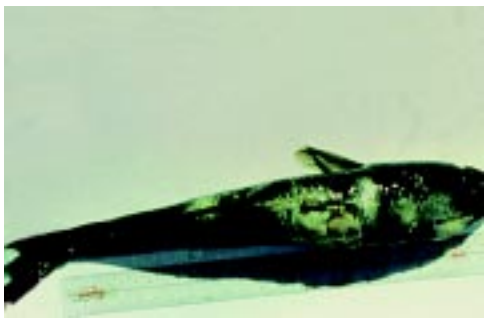

Figure 2-3. Snakehead *(O. striatus)* with EUS lesion

CAUSATIVE AGENT:

This is a complex disease attributed to a combination of rhabdovirus (65x175 nm) (Fig. 2-2), the bacterium *Aeromonas hydrophila*, and the fungus *Aphanomyces invadans*.

SPECIES AFFECTED:

A wide range of cultured and wild fish species are affected namely: snakehead (*Ophicephalus striatus*), catfish (*Clarias* sp.), climbing perch (*Anabas* spp.), spiny eel (*Mastamcembelus armatus*), gourami (*Trichogaster* spp.), barbs (*Puntius* spp.), serpent fish (*Channa micropeltes*), sand goby (*Oxyeleotris marmoratus*), barbs, three-spot gourami (*T. trichopterus*), striped croaking gourami (*Trichopsis vittatus*), siamese fighting fish (*Betta splendens*), wrestling half-beak (*Dermogenus pustillus*), swamp eels (*Fluta alba*). EUS has occurred in Australia, Malaysia, Indonesia, Thailand, Myanmar, Kampuchea, Lao PDR, Philippines, Sri Lanka, Bangladesh, Nepal, Bhutan, Singapore, Vietnam and Pakistan.

GROSS SIGNS:

EUS lesions are characterized by severe, ulcerative, dermal necrosis with extensive erosion/sloughing of the underlying musculature (Fig. 2-3). The necrotic muscular tissue emits a foul odor. Fish manifest frank ulcers consisting of eroded dermal layer, exposing the underlying musculature, which may be hemorrhagic. Fish with less severe lesions exhibit scale loss with erosion of the skin surface with or without hemorrhagic signs.

EFFECT ON HOST:

The histopathological profile of the disease consists of a chronic, necrotic, and mycotic granulomatous inflammatory response.

Outbreaks are observed annually between November to February

when the water temperature is at its lowest. EUS is transmitted by cohabitation with diseased fish or exposure to contaminated waters. The virus replicates at 15 ~ 25°C in 2-3 days to 10^7 $TCID_{50}$/ml in SHS cells and in SSN-1 cells. The virus experimentally induced dermal lesions and mass mortalities of snakehead fry and fingerlings.

DIAGNOSIS:

Signs of disease and isolation/identification of the associated virus, *Aeromonas hydrophila/Aphanomyces* sp., histopathology and electron microscopy.

Channel Catfish Virus Disease (CCVD)

CAUSATIVE AGENT:

Herpesvirus ictaluri (90 to 100 nm)

SPECIES AFFECTED:

Channel catfish (*Ictalurus punctatus*)

GROSS SIGNS:

Acute infection of cultured channel catfish fry and fingerlings less than 10 cm long, juveniles and adults occurs following waterborne exposure to CCV. Clinical signs are abdominal distension (Fig. 2-4), exophthalmia, pale or hemorrhagic gills, petecchial hemorrhage at the base of the fins and throughout the skin. In 20 to 50% of the epizootics, affected fish swim in head-high or hanging position at the surface.

EFFECT ON HOST:

Severe mortality of sometimes nearly 100% occur among young-of-the-year *Ictalurus punctatus* at water temperatures of 25°C or higher within 7 to 10 days. It causes moderate mortalities at 21 to 24°C and almost no mortalities below 18°C. Secondary invasion of external lesions *by Flavobacterium columnares*, *Aeromonas hydrophila* or aquatic phycomycetes may develop. CCVD develops into a hemorrhagic viremia after initially replicating in the kidney and then in the spleen. Thereafter, the virus is transported to the intestine, liver, heart, and brain. Necrosis of the renal hematopoietic tissue and tubules; edema, necrosis and congestion of the liver; intestinal edema; congestion and hemorrhage in the spleen are characteristic histopathological findings. Skeletal muscle hemorrhage among experimentally infected fish has been observed.

Figure 2-4. Channel catfish (*Ictalurus punctatus*) infected with channel catfish virus (CCV). Note the swollen abdomen

Survivors of experimental CCVD averaged only two-thirds the length and one-seventh the weight of control fish 6 months after a standardized feeding regime. The portal of entry for CCV from water is through the gills and the gut. The virus can be isolated from the kidney of fish with active infections during an epizootic using CCO or BB cells, which develop CPE 24 to 48 h post exposure. Optimum replication was observed at 25 to 30°C. The virus is readily transmitted from fish to fish during an epizootic. In natural and experimental infections, exposed fry die within 3 days of exposure and within 7 to 10 days after the first mortality. The virus also persists in apparently healthy adult broodfish.

DIAGNOSIS:

Electron microscopy (EM), serum neutralization tests, indirect fluorescent antibody technique, nested polymerase chain reaction (PCR) and by a channel catfish virus probe.

Grass Carp Hemorrhagic Disease

CAUSATIVE AGENT:

Aquareovirus (60 to 80 nm)

SPECIES AFFECTED:

Grass carp (*Ctenopharyngodon idellus*), black carp (*Mylopharyngodon piceus*), topmouth gudgeon (*Pseudorasbora parva*), silver carp (*Hypophthalmichthys molitrix)*, Chinese minnow (*Hemiculter bleekeri*) and rare minnow (*Gobiocypris rarus*)

GROSS SIGNS:

Clinical signs include exophthalmia, hemorrhagic or pale gills and hemorrhagic fin bases or gill covers.

EFFECT ON HOST:

This disease was first observed in China more than 20 years ago. Outbreaks occured in Southern China during the summer at temperatures of 24-30°C. Acute infections cause significant mortalities of up to 80% among fingerlings and sometimes among yearlings. Internally, hemorrhages occur in the musculature, oral cavity, intestinal tract, liver, spleen and kidneys. Naturally and experimentally infected fish manifest reduced erythrocytes, plasma protein, calcium and urea nitrogen. Serum potassium elevated. Signs of disease and mortality are observed within 1 to 2 weeks of exposure of fish in water at temperatures of 25°C or higher. Experimental vaccination induced 80% level of immunity by day 4 at temperatures above 20°C.

DIAGNOSIS:

The virus can be propagated in cell cultures of GCK-84, GCG and GCF yielding titers as high as 10^8 to 10^9 $TCID_{50}$ per ml. In vitro replication is considered optimum between 28 and 30°C inducing CPE in 3 to 4 days post inoculation. Reverse transcription-polymerase chain reaction (RT-PCR) and electron microscopy are also used for detecting the virus.

Spinning Tilapia (ST) Syndrome

CAUSATIVE AGENT:

Iridovirus (110-140 nm)

SPECIES AFFECTED:

Oreochromis mossambicus, O. aureus, O. niloticus, and *Sarotherodon galilaeus*

GROSS SIGNS:

Affected tilapia fry and fingerlings swim in a spiral pattern, sink to the bottom then rise and hang at a 45 degrees angle just under the water surface, gasping for air. They do not feed and are darker in color.

EFFECT ON HOST:

Tilapia manifesting the spinning syndrome die within 24 h of onset. Up to 100% mortality in affected population of Tilapia fry has been reported. Histopathologically, the renal tubules are shrunken, hemorrhaging and infiltrated with eosinophilic granular cells. In addition, focal myolysis develops in the muscles.

DIAGNOSIS:

The disease is detected by signs of the disease, EM, isolation/identification of the virus.

Viral Nervous Necrosis (VNN)

CAUSATIVE AGENT:

Nodavirus (20-25 nm)

SPECIES AFFECTED:

Grouper (*Epinephelus* spp.), sea bass (*Lates* spp.) (Fig. 2-5), barfin flounder *(Verasper moseri)*, European bass *(Dicentrarchus labrax)*, parrotfish *(Oplegnathus fasciatus)*, striped jack *(Pseudocaranx dentex)*, turbot *(Scophthalmus maximus)*, Japanese flounder *(Paralicthys olivaceus)*, barfin flounder *(Verasper moseri)*, red sea bream *(Pagrus major)*, sea bream *(Sparus aurata)*, shi drum *(Umbrina cirrosa)*, cod *(Gadus macrocephalus)*, Atlantic halibut *(Hippoglossus hippoglossus)*, purplish amberjack *(Seriola dumerili)*, and tiger puff *(Takifugu rubripes)* in Thailand, Japan, Taiwan, Singapore, Tahiti, Greece, Australia and Europe.

GROSS SIGNS:

Affected larvae and juveniles show lethargy, pale color, loss of appetite, thinness, loss of equilibrium and corkscrew swimming. Some fish sink to the bottom then float to the surface again.

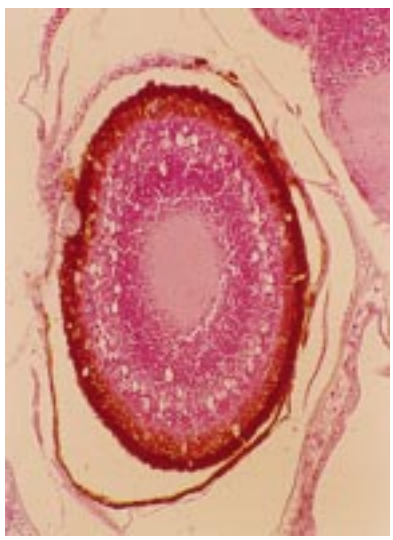

Figure 2-5. Seabass larva: eye with vacuolation (Hematoxylin and Eosin, 40x)

EFFECT ON HOST:

This viral infection is also known as Paralytic Syndrome, Viral Encephalopathy and Retinopathy, Spinning Grouper Disease, Piscine Neuropathy or Fish Encephalitis. The disease is more severe in less than 20 days old larvae. Diseased fish had pale livers, empty digestive tracts, the intestines filled with greenish to brownish fluid and the spleens are red-spotted. The virus replicates in the eye, the brain, and the distal spinal cord of affected fish causing vacuolating encephalopathy and retinopathy. It also multiplies in the gonad, liver, kidney, stomach and intestine. This disease caused 50-95% mortalities among fish larvae and juveniles at 26-30°C in Taiwan and Thailand. It can be transmitted from diseased to healthy fish within 4 days of contact. The virus is more virulent at 28°C than at 16°C. Fish broodstocks can be virus reservoirs.

DIAGNOSIS:

Histopathology showing vacuolations in the nerve cells of the eye retina and the brain of affected fish is diagnostic for this disease. The virus can be isolated in SSN-1 and barramundi cell lines on which it induces CPE. The virus can be identified by EM, PCR, RT-PCR, ELISA, FAT and by *in-situ* hybridization assay.

Lymphocystis Disease

CAUSATIVE AGENT:

Iridovirus (130-330 nm)

SPECIES AFFECTED:

Lates sp. and *Siganus* sp.

GROSS SIGNS:

Infected fish have clusters of pear-like nodules up to 5 mm in diameter that develop on the skin, gills or fins resulting from an enlargement of tissue cells.

EFFECT ON HOST:

The disease is rarely fatal in older fish. It can be transmitted by cohabitation and exposure to contaminated water.

DIAGNOSIS:

Signs of the disease, histopathology and EM

Grouper Iridovirus of Taiwan Disease (TGIV)

CAUSATIVE AGENT:

Iridovirus (220-240 nm)

SPECIES AFFECTED:

Grouper, *Epinephelus* sp.

GROSS SIGNS:

Diseased fish swim in circles and are anemic. Fish lose appetite then become underweight and lethargic. The spleen of affected fish has abnormal hypertrophied cells containing numerous icosahedral virions.

EFFECT ON HOST:

This virus has antigenic similarities with the red seabream iridovirus isolated in Japan, the epizootic, haematopoietic necrosis virus and the iridovirus isolated from sheatfish and the grouper iridovirus isolated in Thailand.

The disease affects farmed groupers, 5-8 cm in total length at 25-28°C. Acute disease causes up to 60% mortality. Experimentally infected fish reach a cumulative mortality of 100% in 11 days without other clinical signs.

DIAGNOSIS:

Signs of the disease; EM

Sleepy Grouper Disease (SGD)

CAUSATIVE AGENT:

Iridovirus (130-160 nm)

SPECIES AFFECTED:

Epinephelus tauvina

GROSS SIGNS:

Affected fish exhibited extreme lethargy and low appetite. Affected fish swim alone or hung at the water surface or remain at the bottom.

EFFECT ON HOST:

It was first reported among farmed groupers, 100-200 g and 2-4 kg in size, in 10 of 33 farms in Singapore and Malaysia. Acute disease causes up to 50% mortality mostly occurring during the night or in the early hours of the morning. Gradual mortalities follow after fish become sluggish over 3-5 days, after which affected fish lie at the net or tank bottom exhibiting weak fin movements. Some terminally ill fish display gill pallor, rapid opercular movements and frantic dashing to the water surface to gulp air. Acute mass mortalities may occur 12-24 h after handling or excessive feeding. Internal pathology consists of enlargement of the spleen or occasional enlargement of the anterior kidney and heart inflammation. The virus was detected in the spleen, heart and kidney of infected fish. Experimentally exposed fish develop signs of SGD and die in 3-4 days. The virus was introduced into a farm with some imported fish and subsequently spread to neighboring farms.

MAJOR VIRAL INFECTIONS IN PENAEID SHRIMPS

White Spot Syndrome Virus (WSSV) Disease

CAUSATIVE AGENT:

Baculovirus (100-140 x 270-420 nm)

SPECIES AFFECTED:

All stages of shrimps like *Penaeus monodon, P. chinensis, P. indicus, P. penicillatus, P. japonicus, Metapenaeus ensis, P. aztecus, P. duorarum, P. merguiensis, P. semisulcatus, P. stylirostris, P. vannamei, P. curvirostris, P. setiferus,* and also other crustaceans such as *Scylla serrata, Charybdis feriatus, Helice tridens, Calappa lophos, Portunus pelagicus, P. sanguinolentus, Acetes* sp., *Palaemon* sp., *Exopalaemon orientalis, Panulirus sp. Macrobrachium rosenbergii, Procambarus clarkii, Orconectes punctimanus, Artemia*

GROSS SIGNS:

Figure 2-6. *Penaeus monodon* affected with the white spot syndrome virus (WSSV)

Typical signs of disease is the presence of distinct white cuticular spots (Fig. 2-6) (0.5-3 mm in diameter) most apparent at the exoskeleton and epidermis of diseased shrimp about 2 days after onset. The white spots start at the carapace and 5th and 6th abdominal segments that later affect the entire body shell. The moribund shrimp display red discoloration and have loose cuticle. Affected shrimps manifest surface swimming and gathering at pond dikes with broken antennae.

EFFECTS ON HOST:

This disease has been reported with the following names: White spot baculovirus (WSBV), White spot virus (WSV), Systemic ectodermal and mesodermal baculo-like virus (SEMBV), Chinese baculovirus (CBV), Hypodermal and hematopoietic necrosis baculo-like virus (HHNBV), Rod-shaped virus of *Penaeus japonicus* (RV-PJ), Penaeid acute viremia (PAV), Penaeid rod-shaped Dovavirus (PRDV). Reduc-

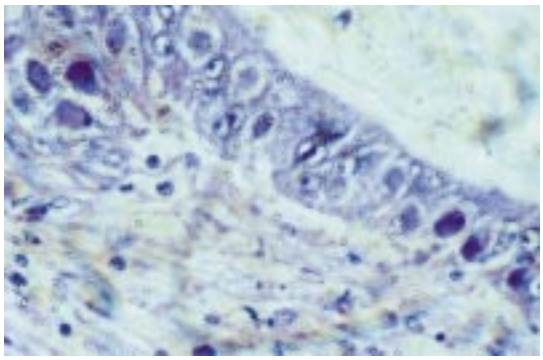

Figure 2-7. Histological section of the stomach of a juvenile *P. monodon* with WSSV intranuclear inclusion bodies (Hematoxylin and Eosin stain, 400x)

tion in food consumption and empty gut develops followed by a rapid onset of the disease and high mortalities of up to 100% in 3 to 10 days. This disease affects a wide host range of crustaceans and targets various tissues (pleopods, gills, hemolymph, stomach, abdominal muscle, gonads, midgut, heart, periopods, lymphoid organ, integument, nervous tissue and the hepatopancreas) resulting in massive systemic pathology. Shrimps, 4-15 g, are particularly susceptible but the disease may occur from mysis to broodstock. Pre-moulting shrimps are usually affected. *Penaeus indicus* suffers earlier and greater losses compared to *P. monodon*. Crabs, krill and other shrimps are viral reservoirs. Pandemic epizootics have occurred in extensive, semi-intensive and intensive culture systems regardless of water quality and salinities.

DIAGNOSIS:

Clinical signs are diagnostic for this disease. However, recent reports indicate that some bacteria may induce similar signs, hence confirmation with other diagnostic tests should be done. Demonstration of the presence of hypertrophied nuclei in stained squashes, smears of epithelial and connective tissues of the gills or stomach of affected shrimp. Histological sections show widespread cellular degeneration and severe nuclear hypertrophy, chromatin margination and eosinophilic intranuclear inclusions in the subcuticular epithelium of the shell, gill, stomach, connective tissues, hematopoietic tissues, lymphoid organ, antennal gland and nervous tissues (Fig. 2-7). Electron microscopy, PCR, DNA probe, Western Blot, and infection bioassay are confirmatony diagnostic tests.

Yellow Head Virus (YHV) Disease

CAUSATIVE AGENT:

Rhabdovirus (40-50 x 150-170 nm)

SPECIES AFFECTED:

Subadults and broodstock of *P. monodon*, *P. aztecus*, *P. duorarum*, *P. merguiensis*, *P. setiferus*, *Palaemon styliferus*, *Acetes* spp.

GROSS SIGNS:

Infected shrimps show light yellowish, swollen cephalothorax. The gills appear whitish, yellowish or brown.

EFFECT ON HOST:

Before the appearance of clinical signs of disease, the shrimps develop an abnormally high feed intake and rapid growth. Thereafter, there is marked reduction in food consumption prior to cessation of feeding and the onset of rapidly accelerating mortality. Moribund shrimps swim slowly near the surface at the edge of the pond. Acute epizooties occur in juvenile to sub-adult shrimps about 20 days post stocking especially during the 50-70 days grow-out culture period. The occurrence of this disease may be associated with unstable phytoplankton bloom, bad pond bottom, high stocking density or exposure to pesticides.

Systemic infection is associated with virus assembled in the cytoplasm of ectodermal and mesodermal cells (gills, lymphoid organ, hemocytes and connec-

tive tissues). Massive necrosis is attributed to cytoplasmic replication of the virus. The virus can cause a total crop loss within 3 to 5 days of onset of clinical signs with incubation period of 7-10 days. The virus in water remain infective up to 72 h. Shrimp reservoirs include *Palaemon styliferus*. About 4% broodstock are infected. In the Philippines, a recent sampling of 250 shrimps reported positive for YHV in 16% of specimens.

DIAGNOSIS:

Signs of disease and phase contrast microscopy of fresh hemolymph stained with Wright/Giemsa stain. Histopathological analyses show the presence of basophilic usually spherical, perinuclear cytoplasmic inclusions in the hemocytes, lymphoid organ, hematopoietic tissues, pillar and epithelial cells in the gills, spongy connective tissue cells in the subcutis, muscle, gut, antennal gland, gonads, nerve tracts, ganglia and other cells of ectodermal and mesodermal origin. Electron microscopy, Western blot, RT-PCR, and infection bioassay are confirmatory diagnostic tests.

Monodon Baculovirus (MBV) Disease

CAUSATIVE AGENT:

P. monodon-type baculovirus (75 x 300 nm)

SPECIES AFFECTED:

The giant tiger prawn *Penaeus monodon*, and other penaeid shrimps like *P. merguiensis, P. vannamei, P. esculentus, P. semisulcatus, P. penicillatus, P. plebejus, P. kerathurus*.

GROSS SIGNS:

Affected shrimps exhibit pale-bluish-gray to dark blue-black coloration, sluggish and inactive swimming movements, loss of appetite and retarded growth. An increased growth of benthic diatoms and filamentous bacteria may cause fouling on the exoskeleton/gills. Infected pond-reared shrimps at 45 days of culture (DOC) stocked at 4 to 100 per m^2 manifested slow growth rates and pale yellow to reddish brown hepatopancreas.

EEFFECT ON HOST:

This is among the first viral infections diagnosed in mysis, postlarvae, juveniles and adults of the giant tiger prawn, *Penaeus monodon*. The virus causes destruction of the hepatopancreas and lining of the digestive tract. Spherical, eosinophilic occlusion bodies fill up enlarged nuclei of hepatopancreatic cells and are discharged into the lumen after cells have been destroyed. This may be followed by necrosis with secondary bacterial infection. PL-3 is the earliest stage found infected with MBV. However, experimental waterborne inoculation of MBV to mysis-2 (M-2), postlarvae-3 (PL-3), PL-6, PL-9 and PL-11 resulted in MBV infections within 12 days post-inoculation. The incidence rate of MBV was reported at 20-100%. Cumulative mortality of 70% was observed among *P. monodon* juveniles cultured in raceways and tanks. It is associated with a high incidence of bacterial infections expressed as localized “shell disease.” In addition, significant mortalities can occur during stress and crowding.

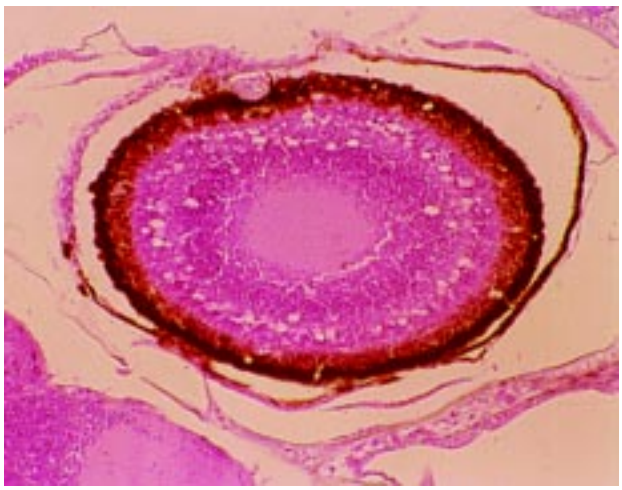

Figure 2-8. Squash preparation of the *Penaeus monodon* hepatopancreas showing normal cells and occlusion bodies of Monodon Baculovirus (MBV) (arrow) (Malachite green stain, 400x)

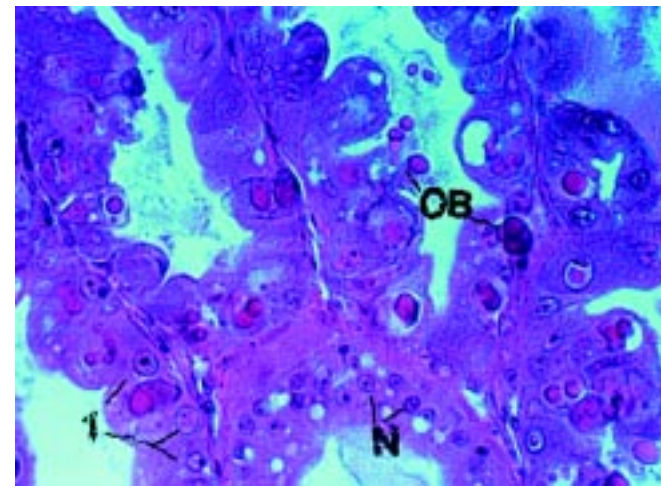

Figure 2-9. Histological section of the *Penaeus monodon* hepatopancreas with occlusion bodies (OB) of Monodon Baculovirus (MBV) (Hematoxylin and Eosin, 400x)

DIAGNOSIS:

Demonstration of occlusion bodies in wet mounts of feces, midgut or hepatopancreas stained with malachite green (Fig. 2-8). Histological sections show the presence of eosinophilic, multiple occlusion bodies within the hypertrophied nuclei of the hepatopancreatic tissues with the following development (Fig. 2-9). Other diagnostic tests are DNA probe and PCR.

Stages of Cytopathology

Stage 0 Cell infected by MBV but cytopathic changes not yet apparent

Stage 1 Slight hypertrophy of the nucleus, chromatin margination, peripheral migration of nucleolus. Viral replication is initiated.

Stage 2 Increased nuclear hypertrophy, proliferation of the virus and development of eosinophilic occlusion bodies.

Stage 3 Hypertrophied nucleus up to twice the normal diameter and six times the normal volume. One or more occlusion bodies and abundant virions are present

Infectious Hypodermal and Hematopoietic Virus (IHHNV) Disease

CAUSATIVE AGENT:

Parvovirus (20-22 nm)

SPECIES AFFECTED:

Postlarvae, juveniles and adults of *P. monodon, P. stylirostris, P. vannamei, P. semisulcatus, P. schmitti, P. setiferus, P. aztecus, P. duorarum, P. californiensis* and *P. japonicus*.

GROSS SIGNS:

Shrimps show erratic swimming behavior, rising slowly to the water surface, hanging and rolling over until the ventral side is up. Eventually, the animal

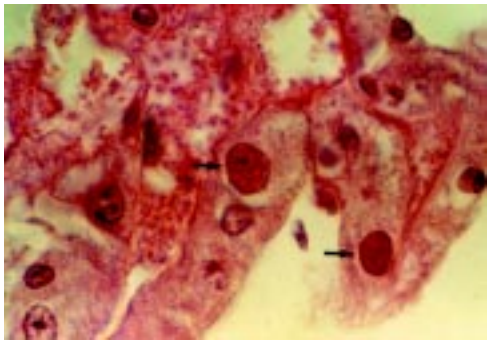

Figure 2-10. Histological section of *P. monodon* and antennal gland showing intranuclear inclusion bodies of IHHNV (arrows) (Hematoxylin and Eosin, 1000x)

sinks to the bottom. Shrimps would eventually right themselves up, become weak and lose their appetite for food. They repeat the process of rising to the surface and sinking until they die usually within 4-12 h. The shrimps manifests decreased preening and delayed molting. Acutely affected shrimps develop white opaque abdominal muscles, bluish to distinctly blue cuticular color often with mottled buff to tan pigment patches in the cuticular hypodermis and very soft cuticles.

EFFECT ON HOST:

They have poor resistance to stress. Mortality rates of above 90% were observed among penaeid juveniles in intensive culture systems. The virus infects cells of the ectodermal tissues (epidermis, hypodermal epithelium of foregut and hindgut, nerve cord and nerve ganglia) and mesodermal tissues (hematopoietic organs, antennal gland, connective and striated muscles, heart, gonad, mandibular organ, hemocytes). It induces the development of eosinophilic inclusion bodies in the cytoplasm of affected cells during the early acute stage of the disease, followed by necrosis and inflammation of target tissues. The presence of the virus can cause death of the cells of the cuticle, blood-forming tissues and connective tissues of the shrimp that leads to abnormal metabolism and eventually mortalities. Inclusion bodies are common early in acute infections, later decreasing in number followed by necrosis and inflammation of target tissues.

The disease can be experimentally induced in *P. setiferus, M. japonicus, P. aztecus* and *P. duorarum*. It has been linked to the Runt deformity syndrome in *L. vannamei*. Secondary bacterial infection may occur. Some survivors of epizootics may carry the virus for life.

DIAGNOSIS:

Histological demonstration of eosinophilic intranuclear inclusion bodies (Fig. 2-10) in the hepatopancreas by H & E staining. Electron microscopy, PCR, DNA probe, infection bioassay are other diagnostic tests.

Hepatopancreatic Parvo-like Virus (HPV) Disease

CAUSATIVE AGENT:

Parvovirus (22-24 nm)

SPECIES AFFECTED:

Postlarvae, juveniles and adults of *P. monodon, P. merguiensis, P. vannamei, P. esculentus, P. semisulcatus, P. penicillatus, P. indicus, P. chinensis*.

GROSS SIGNS:

Affected shrimps develop loss of appetite and retarded growth. Benthic diatoms, protozoans such as *Zoothamnium* sp., and filamentous bacteria may cause fouling on the exoskeleton of infected shrimps. Occasionally, white opaque areas on the tail/abdominal muscles are observed.

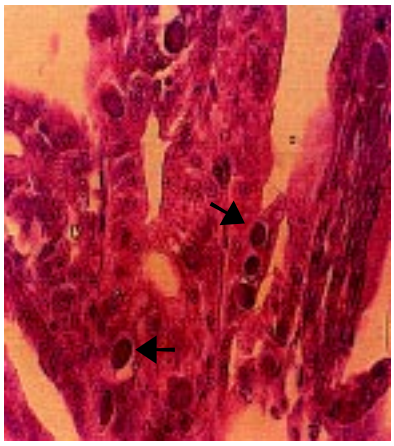

Figure 2-11. Histological section of the hepatopancreas of *Penaeus monodon* infected with the Hepatopancreatic Parvo-like virus (HPV). Arrows point to occlusion body (Hematoxylin and Eosin, 400x)

EFFECT ON HOST:

Postlarvae (PL-1–PL-19) from three hatcheries in Iloilo showed prevalence rates of 7.8 to 26.4%. Mortalities among *P. merguiensis* may reach as high 50% within 4-8 weeks of disease onset. The virus cause hypertrophy of the hepatopancreatic nucleus with lateral displacement and compression of the nucleolus and chromatin margination with development of a prominent basophilic occlusion body. This leads to cell death and shrinkage of the hepatopancreas. Damage of this organ can result in abnormal metabolism and eventually death.

DIAGNOSIS:

Signs of disease and histological demonstration of single prominent basophilic (H&E stain) intranuclear occlusion body in the hypertrophied nucleus of the hepatopancreatic cell (Fig. 2-11) are diagnostic. Electron microscopy, DNA Probe, PCR, infection bioassay are confirmatory tests.

PREVENTION OF VIRAL INFECTIONS

There are no treatments for viral infections in fish or shrimps. Hence, preventive measures must be adapted to keep the viral pathogens away. The basic consideration in preventing the occurrence of viral diseases is avoidance, and the use of virus-free fry for stocking in ponds is highly recommended. It should also be borne in mind that semi-intensive and intensive culture systems promote conditions conducive for disease development. As such, reduction of stress and the application of good husbandry or efficient technology may deter the occurrence of disease. Details on the general methods of disease prevention are discussed in Chapters 9 and 10.

In addition, specific precautions of egg washing with ozone-disinfected seawater; using fine screens for inlet water and adherence to strict hygiene; stress test of shrimp postlarvae (PLs) with 100 ppm formalin for 30 min with aeration and stocking only tolerant PLs; the use of only dry commercial feeds and pasteurized fresh feed at 60°C for 15 min before use; and feeding with feeds containing 100 ppm phosphated ascorbic acid (MAP) for 92 days were reported effective for viral infections. For WSSV management, the effective use of immunostimulants mixed with feed such as peptidoglycan (PG) at 0.2 mg/kg body weight/day for 2-3 months or Fucoidan, an extract from *Cladosiphon okamurans* at 50-100 mg/kg shrimp/day for 15 days, were reported to increase survival of WSSV-exposed shrimps.

It is also important that surveillance for early signs of disease and stressful factors become essential components of farm/hatchery management in order to monitor the health status of fish stocks, to assess adequacy of rearing procedures and to prevent introduction of pathogens. Early detection of a disease

outbreak will reduce mortalities and prevent a catastrophic spread of the virus. Finally, since virus can remain viable outside the living host for at least 72 hr, water change should be contemplated only at least 5 days after effluents from infected ponds in the area have been discharged.

SUMMARY

Outbreaks of viral infections can cause massive mortalities among cultured fishes or shrimps. Water temperature and age of the fish or shrimps are significant factors that influence the development of viral infections. Most fish viral infections occur at low water temperatures, hence, very few viral infections among fishes in warm water culture systems are reported. In addition, most viral infections occur among fry or fingerlings often causing severe mortalities, while older fish or shrimp develop resistance or are hardly affected. Stress from handling, poor water quality, high stocking density and poor nutrition also affect the severity of viral infections.

Finally, aquaculturists should beware in importing non-indigenous fish or shrimps into the country as these are potential carriers of viral pathogens.

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3

CHAPTER THREE Bacterial diseases

Eleonor V. Alapide-Tendencia and Leobert D. de la Peña

Diseases caused by bacteria may cause heavy mortality in both wild and cultured fish and crustaceans. Bacteria are found everywhere in the aquatic environment. Most bacterial disease agents are part of the normal microflora of the marine environment and are generally considered as secondary or opportunistic pathogens. Almost all fish bacterial pathogens are capable of independent existence outside the fish. There are only a few obligatory pathogens. Even these, however, are capable of living for a long time in the tissues of their host without causing injury. Clinical infections and disease usually occur only after the onset of some major changes in the physiology or body of the host. Thus, to understand bacterial diseases of fish, one must understand the relationship of bacteria with their host and with their environment.

As in all animal production systems, bacterial disease is one of the major problems facing production, development and expansion of the aquaculture industry. The control of disease is particularly difficult because fish are often farmed in systems where production is dependent on natural environmental conditions. Changes or deterioration in the aquatic environment cause most of the bacterial diseases encountered, and environmental effects give rise to many other adverse conditions. A second major constraint on disease control is the relatively limited number of therapeutic agents available for the control of bacterial disease agents. Even recommended therapies and preventive measures pose limitations. Their application to aquatic animals is often difficult in actual practice, and sometimes impossible to implement.

Outbreaks of major bacterial diseases in aquaculture can be significantly reduced if proper attention is paid to good husbandry practices and the maintenance of optimum environmental conditions, especially water quality. Another important consideration involves identifying the predisposing factors that may lead to a disease state. Once predisposing factors are identified, appropriate corrective measures should be initiated in the culture system.

Most bacterial disease show similar signs, especially in fishes. Bacterial infection may appear on the skin or fins of fish, exoskeleton or appendages of crustaceans, in the muscles and in the internal organs. In nearly all cases, red spots, brown or black spots, or necrotic tissues can be observed. Inflammation may also occur. Proper identification of the causative agent is important to ensure successful treatment.

WHAT ARE BACTERIA?

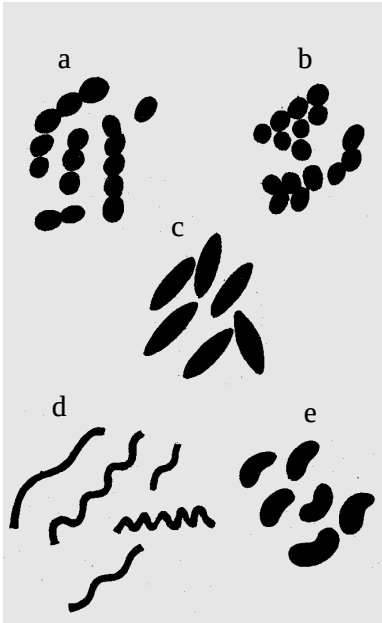

Figure 3-1. The different shapes and arrangements of bacteria: (a) cocci in streptococcal arrangement, (b) cocci in staphylococcal arrangement, (c) bacilli, (d) several kinds of spirilla, and (e) comma-shaped vibrios

Bacteria are not visible to the naked eye. These microorganisms are of very small dimensions, usually between 0.5 and 10 microns (µm). But, when bacteria multiply in great numbers on a solid medium, they form visible colonies representing millions or billions of individual cells. The cells can be seen only under a microscope from a smear stained with a dye on a microscope slide.

Bacteria differ from other cells in that they are prokaryotic (lacking a nuclear membrane). The nucleus occupies the center of the cell. All its genetic material is linked in a single chromosome. The cytoplasm is densely packed with RNA and is finely granular because of the presence of ribosome. The nucleus/ cytoplasm complex is packaged in a complex envelope or integument. Its innermost layer is the thin cytoplasmic membrane (plasmalemma). Outside the membrane is a rigid cell wall. Some bacterial pathogens develop a capsule outside of the cell wall, which is usually associated with the virulence or infective ability of the organism. Many of the pathogenic bacteria are flagellated and a few have no flagella for locomotion. Some move by body flexing or gliding.

Some bacteria produce enzymes called extracellular products or ECP, which are associated with the microorganism's virulence. Extracellular products are highly toxic to fish and crustaceans and they contain proteases, hemolysins, exohemagglutinins and cytotoxins.

Bacteria reproduce asexually by binary fission. That is, they multiply by an elongation of the cell followed by a division.

The most common method used to detect the presence of bacteria is by gram stain. The gram stain classifies bacteria into two groups: the gram positive and the gram negative. Gram-positive bacteria are those that possess a thick peptidoglycan cell wall which will retain the initial crystal violet stain during washing with 95% alcohol. Gram-negative bacteria are those that possess a unimolecular peptidoglycan cell wall bounded on one side by the cytoplasmic membrane and on the other side by the outer membrane; such cells are decolorized by 95% alcohol and take up the secondary stain. To identify bacterium, a pure culture should be obtained, containing a single species and not a mixture of different kinds of bacteria.

In classifying bacteria, one needs to pay attention to the cell shape. There are three distinct cell forms: cocci, baccili and spiral (Fig.3-1). Cocci are spherical cells and exist in several patterns or groupings which are specific to the genus. Paired cocci are called diplococcus, while those in long chains are called streptococcus. Irregularly grouped cocci are called staphylococcus. Longer and cylindrical bacteria are known as bacilli or rods. Cells that are between the coccus and the bacillus in shape are called coccobacilli. The short, curved rods are the vibrios. When more than one curvature is observed, it is called spirilla. Most bacteria that cause disease in fish and crustaceans are rod-shaped. Figure 3-1 shows the different shapes and arrangements of bacteria.

The shape, size and color of a given colony are also important in identification. The bacterial colony surface texture, whether rough, smooth or mucoid, should

also be observed. The same species can form rough or smooth colonies, depending on environmental conditions and the virulence or infective ability of the strain. Smoother colonies are often more virulent or harmful. Considering the large number of bacteria known to exist, gram stain reaction and morphological form are not enough to identify bacterial species. To identify a bacterium, the different physiological or bodily and biochemical properties it possesses must first be known through a series of tests.

IDENTIFYING THE REAL CAUSE OF A DISEASE; KOCH'S POSTULATES

Not all bacteria present in the body of a fish or crustacean are pathogenic or may cause disease. Some bacteria may be harmless or even beneficial. By carefully noting the observations suggested here, one might be able to tell if the isolated bacterium suspected of causing the disease is the causative organism.

Koch, a German physician and bacteriologist, enunciated the following criteria in 1891 to establish unequivocally a causal relationship between an organism and a specific disease.

- The organism should be found in all cases of the disease in question, and its distribution in the body should be in accordance with the lesions observed.
- The organism should be cultivated outside the body of the host, in pure culture, for several generations.
- The organism so isolated should reproduce the disease when introduced into other susceptible animals.
- The organism must be reisolated from the experimentally-infected animal.

Virulence Determinants

To produce disease, microorganisms must be able to:

- Enter the host;
- Multiply under the physical and chemical conditions of the host tissues;
- Interfere with the action of humoral (in body fluids) and cellular defense mechanisms of the host; and
- Damage tissues thereby producing the unpleasant and possibly lethal effects.

Bacteria are ubiquitous. This means that they can be found or are present almost everywhere in the aquatic environment. The actual role of these microorganisms may vary from being beneficial to that of being a secondary opportunistic invader, attacking only when the host is weakened or injured, or a primary pathogen that may cause the death of the species.

In this chapter, the important bacterial diseases of fish and crustaceans are discussed as well as the different diagnostic methods, gross signs and preventive measures and treatments of these diseases.

IMPORTANT BACTERIAL DISEASES OF FISH

Columnaris Disease

CAUSATIVE AGENT:

Flavobacterium columnare (previously named as *Flexibacter columnaris*)

SPECIES AFFECTED:

Ayu *(Plecoglossus altivelis)*, tilapia *(Oreochromis niloticus)*, carp *(Cyprinus* sp.*)*, channel catfish *(Ictalurus punctatus)*, goldfish *(Carassius auratus)*, rohu *(Labeo rohita)*

GROSS SIGNS:

The first indication of infection is generally the appearance of a white spot on some part of the head, gills, fin or body. A zone with a distinct reddish tinge usually surrounds this. The lesions are circular as if spreading from a single focus towards all directions at the same rate. On the gills, the lesions are more necrotic. On the skin, they develop into hemorrhagic ulcers, with an overlying seroma of bacterial cell and necrotic tissue. Histologically, there is epidermal spongiosis, necrosis, and ulcerations.

EFFECTS ON HOST:

F. columnare is an opportunistic pathogen widely distributed in the water. The disease does not usually occur as spontaneous infection but results from injuries to the fish, or physical and nutritional deficiencies. Outbreaks are affected by factors such as temperature and stress. Dissolved cations such as sodium, potassium, calcium and magnesium enhance their infectivity. *F. columnare* attacks fish primarily through the gills or abraded epidermal areas. Once the pathogen is established, proteolytic enzymes break down the skin and muscle to open necrotic lesions. The bacterium appear systematically after extensive tissue necrosis. Gill lesions may cause respiratory difficulty and the fish eventually dies. Body lesions are subject to secondary infection by other microorganisms. Fish that survive the infection may become carriers of the disease.

DIAGNOSIS:

Columnaris disease can be presumptively diagnosed from disease signs on the skin and gills of the host and from squash preparations made from scrapings of the affected areas. In wet mount preparations of infected tissues, the bacteria show a slow gliding movement, gathering into characteristic column-like masses that give the disease its name. Microscopic examination of lesions shows the presence of long, slender, possibly filamentous, rod-shaped, gram-negative bacteria. The growth of *F. columnare* on solid media is usually characterized by yellow-green, flat and rough spreading colonies that adhere to the media. *F. columnare* can also be detected from fish and water using indirect fluorescent antibody technique (IFAT).

PREVENTION AND CONTROL:

- vaccination
- environmental manipulation like lowering water temperature
- addition of competitive bacteria like *Citrobacter fecundii* and *Aeromonas hydrophila*
- copper sulfate dip at 40 mg/L for 20 min or 500 mg/L for 1 min
- oxolinic acid dip at 1 mg/L for 24 h
- sulphamerazine and oxytetracycline at 220 mg/kg/day for 10 days followed by 50 to 75 mg/kg/day for 10 days.

Edwardsiella Septicaemia or Edwardsiellosis

CAUSATIVE AGENT:

Edwardsiella tarda

SPECIES AFFECTED:

Tilapia *(Oreochromis niloticus)*, channel catfish *(Ictalurus punctatus)*, mullet *(Mugil cephalus)*, carp *(Cyprinus carpio)*

GROSS SIGNS:

Edwardsiella tarda infection manifests itself by the presence of small, 3-5 mm cutaneous or skin lesions located dorso-laterally (from along the back to the side) on the body. These lesions progress into abscesses and develop obvious convex swollen areas. The skin loses pigmentation. A foul smelling gas is emitted when the skin is incised. The lesion contains large amounts of necrotized or dead tissue. Internally, there is generalized hyperemia (Fig. 3-2) and enlargement of the liver and kidney. Histologically, the lesion is characterized by focal necrosis, often extending from muscle, haemopoietic tissue and liver parenchyma (Fig. 3-3) to perforate the abdominal wall.

Figure 3-2. *Oreochromis niloticus* with *Edwardsiella tarda* infection showing white nodules on the liver

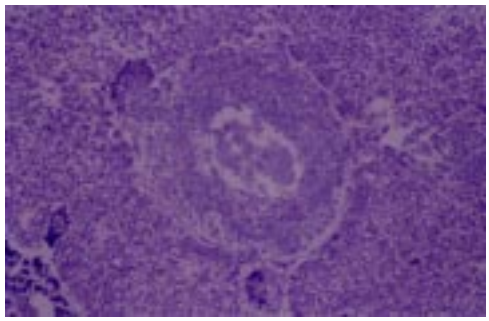

Figure 3-3. Focal necrosis in the liver of *O. niloticus* infected with *Edwardsiella tarda*. (Hematoxylin and Eosin stain, 25X)

EFFECTS ON HOST:

E. tarda infection usually occurs during the warm, summer months. The source of *E. tarda* is presumably the intestinal contents of carrier animals such as snakes, fish (eel and catfish), and some amphibians and reptiles. High temperature, poor water quality and crowding may contribute to the onset and severity of the disease. Affected fish lose mobility of the caudal or tail portion of the body. *E. tarda* infection may cause lesions in the dermis, musculature and visceral organs of the host. Skin lesions when incised emit a foul smelling gas. The lesion contains large amount of necrotized or dead tissue.

DIAGNOSIS:

The bacterium is easily isolated from muscle and internal organs of clinically diseased fish on most general-purpose media such as brain heart infusion agar (BHIA) and tryptic soy agar (TSA). Small punctate colonies develop in 24-48 h on inoculated media.

PREVENTION AND CONTROL:

- Improve water quality.
- Reduce stocking density.
- Apply oxytetracycline at 55 mg/kg fish for 10 days.

Vibriosis

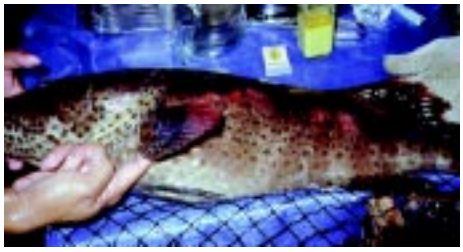

Figure 3-4. *Vibrio* infected grouper with hemorrhagic and necrotic fins. Hemorrhagic lesions on the dorsal body part can also be observed

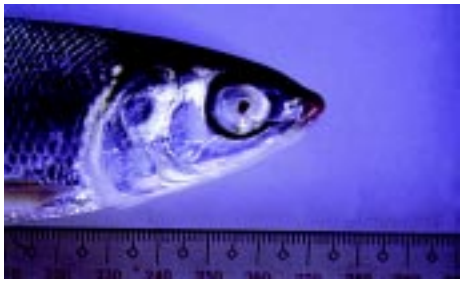

Figure 3-5. Eye opacity in milkfish fingerlings infected with *Vibrio parahaemolyticus*

Figure 3-6 Bilateral exophthalmia in fish with bacterial infection

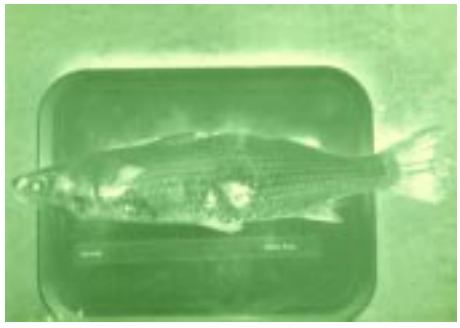

Figure 3-7a. Dermo-muscular lesion in mullet with vibrio infection

CAUSATIVE AGENT:

Vibrio alginolyticus, V. anguillarum and *V. vulnificus*

SPECIES AFFECTED:

Grouper *(Epinephelus* sp.*)*, rabbitfish *(Siganus* sp.*)*, milkfish *(Chanos chanos)*, seabass *(Lates calcarifer)*, sea bream *(Sparus aurata)*

GROSS SIGNS:

The first signs of the disease are usually anorexia or loss of appetite, with darkening either of the whole fish or of particular areas of the dorsum or back. Other common signs of vibriosis are hemorrhagic spot on different parts of the body including necrotic fins (Fig. 3-4), eye opacity (Fig. 3-5) and exophthalmia (Fig. 3-6). The perachute condition results in death without any other clinical signs except occasional periorbital or abdominal oedema. Chronically infected fish generally exhibit very pale gills and large granulating lesions deep in the muscle (Fig. 3-7a; 3-7b). In hatcheries, the presence of red spots in tanks is a sign of *Vibrio* infection.

EFFECTS ON HOSTS:

Vibriosis usually occurs in the warm summer months, especially when the stocking densities are high, and the salinities and organic loads are also high. Stressed fish are more prone to *Vibrio* infection. When an outbreak occurs, mortalities of 50% or higher can be observed in young fish. In older fish, losses may be lower, but infected fish do not feed or grow. When harvested, fish may have large necrotic lesions in the middle of the muscle mass.

DIAGNOSIS:

Squash preparations of kidney, liver, spleen, necrotic muscle tissue and other organs reveal the bacterium. The pathogen can usually be isolated from infected organs in pure culture using standard bacteriological media, such as BHIA, nutrient agar (NA) and TSA, provided they contain 1-2% sodium chloride. Thiosulphate citrate bile salt agar (TCBS) is a medium that selectively promotes growth of pathogenic vibrios while inhibiting other bacteria.

PREVENTION AND CONTROL:

- Maintain good water quality, good husbandry procedures and lower stocking densities.
- Apply oxytetracycline at 77 mg/kg of fish or nitrofurazone at 56 mg/kg of fish for 10 days.
- Vaccinate.

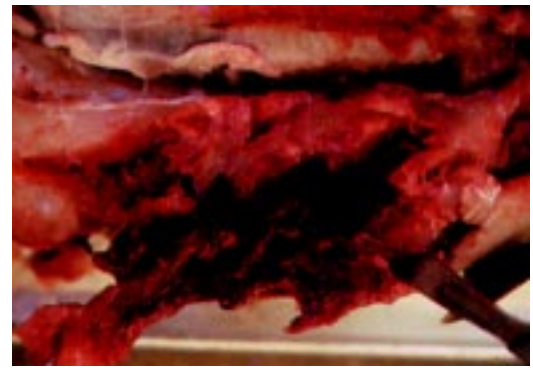

Figure 3-7b. Hyperemia and blood clot in the abdominal cavity of mullet with vibrio infection

Motile Aeromonad Septicemia

CAUSATIVE AGENT:

Aeromonas hydrophila, A. caviae, and *A. sobria.*

Species affected:

Tilapia *(Oreochromis niloticus)*, milkfish *(Chanos chanos)*, goldfish *(Carassius auratus)*, catfish *(Clarias batrachus)*, snakehead *(Ophicephalus striatus)*, goby *(Glossogobius guirus)*, climbing perch *(Anabas testudineus)*, gourami *(Trichogaster* sp.*)*, mullet *(Mugil cephalus)*

GROSS SIGNS:

External signs of motile aeromonad disease vary from darkening in color, enlargement of the abdominal area (Fig. 3-8a) to an extensive superficial reddening of a large area of the body (Fig. 3-9), often with necrosis of fins or tail and extensive ulceration over a considerable portion of the flanks or dorsum. The ulcers are usually shallow and the surface may go brown as it necrotizes or decays (Fig. 3-10). Other disease signs are scale loss, mouth sores,

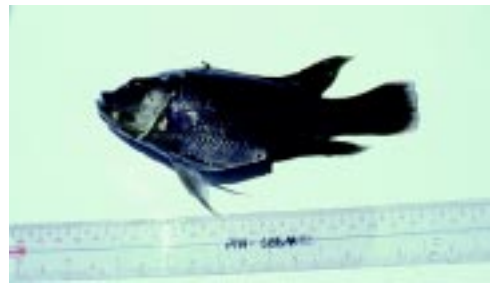

Figure 3-8a. *Oreochromis niloticus* with *Aeromonas hydrophila* infection. Note the enlarged abdominal cavity

Figure 3-8b (far right). The same *O. niloticus* with hyperemia and enlarged abdominal cavity due to *A. hydrophila*

Figure 3-9. Hemorrhagic lesion in milkfish infected with *Aeromonas hydrophila*

Figure 3-10 (far right). Dermo-muscular lesion in catfish experimentally infected with *A. hydrophila* obtained from EUS fish

exophthalmia, and eye opacity. Internally, there may be dropsy (Fig. 3-8b), hyperemia, and the congestion of the internal organs.

EFFECTS ON HOST:

The organisms are usually transmitted through the mouth but may also enter through the skin or gill abrasions. The organisms multiply in the intestine or at the site of invasion and are spread throughout the body by the bloodstream. Internally, there may be ascitic fluid, anemia and damaged internal organs which may lead to mortalities. Mortality as high as 80% may occur among physically stressed, nutritionally deficient, anoxious or injured young fish. Older fishes are less susceptible to motile aeromonads, although 20 to 35% mortalities are not common.

DETECTION AND CULTIVATION:

Squash preparation of the kidney is useful when searching for the etiological agent of the disease. The organisms appear as rod-shaped bacteria, a few are coccoids or short rods in form, usually in single or pairs but rarely in short chains or filaments. They grow well on most common laboratory media such as BHIA, TSA and NA.

PREVENTION AND CONTROL:

- Avoid overcrowding of fish in holding facilities.

Pseudomonad Septicemia or Red Spot Disease

CAUSATIVE AGENT:

Pseudomonas fluorescens, P. anguilliseptica, and *P. chlororaphis.*

SPECIES AFFECTED:

Milkfish *(Chanos chanos)*, goldfish *(Carassius auratus)*, tilapia *(Oreochromis niloticus)*

GROSS SIGNS:

The external disease signs of pseudomonad septicaemia are similar to those caused by other gram-negative bacterial pathogen of fish. The disease causes small hemorrhages in the skin around the mouth and opercula and along the ventral or abdominal surfaces. The body surface may ooze blood and slime in severe cases but there is no reddening of the fins and anus.

EFFECTS ON HOST:

Pseudomonas spp. enters the host either through the oral route or through broken or abraded skin and damaged gills. The organism is carried throughout the fish body by the blood stream. The bacteria and their toxin destroy body tissues, organs and functions. Dysfunctions of the different body organs may lead to mortality of up to 70%.

DIAGNOSIS:

The organisms can usually be isolated from the kidney and other internal organs of affected fish, as well as from the lesion. They grow well on most common laboratory media such as BHIA, TSA and NA. GSP agar or *Pseudomonas-*

Aeromonas Selective Agar is a medium that selectively promotes growth of *Aeromonas* and *Pseudomonas*, and inhibits growth of other bacteria.

PREVENTION AND CONTROL:

- Maintain proper stock management procedures, ensure water quality and reduce stocking density.
- Transfer in a tank and raise temperature to 26-27°C and maintain for about 2 weeks.

Streptococcal Infection

CAUSATIVE AGENT:

Streptococcus sp.

Species affected:

Seabass *(Lates calcarifer)*, tilapia *(Oreochromis niloticus)*, rabbitfish *(Siganus guttatus)*, ayu *(Plecoglossus altivelis)*

GROSS SIGNS:

Clinical signs vary among species of affected fish. However, erratic swimming, darkening of body color, unilateral or bilateral exopthalmia, corneal opacity, hemorrhages on the opercula and the bases of the fins and ulceration of body surface are the most common clinical signs. The hemorrhagic lesions, which gradually extend and ulcerate to release decayed material, are generally raised and have a darkened zone around them. The lesions are more superficial than in furunculosis or vibriosis.

EFFECTS ON HOST:

Infected fish have difficulty ventilating, and lose the ability to orient themselves in the water. The eye becomes opaque and necrotic, conditions that can result to blindness. Fish swim in a spiralling motion. Fish are able to respond to stimuli, but have little control over movements. The spleen and kidney become enlarged. Dysfunction of the damaged internal organs may lead to mortalities.

DIAGNOSIS:

The pathogen grows easily on tryptic soy agar supplemented with 0.5% glucose, brain heart infusion agar, Todd-Hewitt broth agar and horse agar. Colonies develop after 24-48 h of incubation at 20-30°C. The colonies on agar plates appear small (0.5-1mm diameter), yellowish, translucent, rounded and slightly raised.

PREVENTION AND CONTROL:

- Avoid overcrowding, overfeeding and unnecessary handling or transport.
- Remove and slaughter promptly all moribund fish in ponds or net cages at early stage of infection to prevent outbreak or reduce severity of disease.
- Apply erythromycin at 25-50mg/kg body weight of fish for 4-7 days.

Mycobacteriosis or Piscine Tuberculosis

CAUSATIVE AGENT:

Mycobacterium marinum, M. fortuitum and *M. chelonae*

SPECIES AFFECTED:

Siamese fighting fish *(Betta splendens)*

GROSS SIGNS:

Piscine mycobacteriosis is a systemic, chronic, progressive disease presenting various clinical features depending upon species and ecological conditions. Listlessness, anorexia, emaciation, exopthalmia, skin discoloration and external lesions ranging from scale loss to nodules, ulcers and fin necrosis are signs of advancing infection. Gross internal pathology of mycobacteriosis show gray-white lesions of various sizes in most organs and tissues, but the kidney and liver are most often involved.

EFFECTS ON HOST:

Mycobacteriosis is a chronic progressive disease. The disease may take several years to progress from the asymptomatic state to clinical illness. Initially the pigment will fade, and the fish appear sluggish with loss of appetite. Skin ulcers will develop. Fin and tail rot and loss of scale may also be seen. Nodules may form in the muscle and internal organs, which may lead to emaciation or edema or peritonitis. Infection may spread to the skeleton, in which deformities become apparent. Mortalities will then be observed.

DIAGNOSIS:

Primary isolation of fish mycobacteria is best achieved using Ogawa and Lowenstein-Jensen media. Subcultures develop at 28°C within 3-5 days on these media. On Ogawa medium, the cultures appear creamy in the dark but brilliant yellow color when exposed to light. Cultures may not always be obtained even from fish showing unequivocal evidence of infection. Mycobacteria may also be isolated occasionally on general-purpose bacteriological media such as tryptic soy agar, or brain heart infusion agar, provided that a large inoculum is used. All fish mycobacterium have been cultured at 20-30°C for 2 to 30 days. The isolates are strongly acid-fast, rod-shaped, weakly gram-positive, cord forming, non-motile and non-spore forming. Optimum temperature for bacterial growth is between 15°C to 37°C, but the isolates grow best at 28°C.

PREVENTION AND CONTROL:

- Sanitation, disinfection, and destruction of carrier fishes are the primary methods of controlling mycobacteriosis.
- Avoid feeding fish with contaminated fish products. Pasteurize food before use.
- Apply chloramine B or T at 10 mg/l for 24 h.

BACTERIAL DISEASES OF CRUSTACEANS

Bacterial infections of cultured crustaceans occur as: bacterial fouling of surfaces, cuticular or subcuticular localized infections, and internal or systemic infections.

Bacterial Fouling of Surfaces

Filamentous Bacterial Disease

CAUSATIVE AGENT:

Leucothrix sp., *Thiothrix* sp., *Flexibacter* sp., *Cytophaga* sp., *Flavobacterium* sp.

SPECIES AFFECTED:

Penaeus monodon, P. merguiensis, P. indicus

GROSS SIGNS:

Presence of fine, colorless, thread-like growth on the body surface (Fig. 3-11) and gills as seen under a microscope.

EFFECTS ON HOST:

Infected eggs show a thick mat of filaments on the surface, which may interfere with respiration or hatching. In larvae and postlarvae, normal respiration, feeding, locomotion, and molting may be seriously impaired, resulting in slower growth rates, retarded development and eventually death. However, larval shrimps are less prone to infestations due to rapid succession of molts throughout the different larval stages. Frequent molting does not allow adequate time for the bacteria to accumulate on the exoskeleton. In larger shrimps, it may result in respiratory distress. Mortality is due to hypoxia. Disease onset is associated with high organic loads in culture water, low dissolved oxygen levels and added stress from molting. If left untreated in intensive culture systems, accumulative mortality may reach 80% or more within a few days to a few weeks of onset of disease signs.

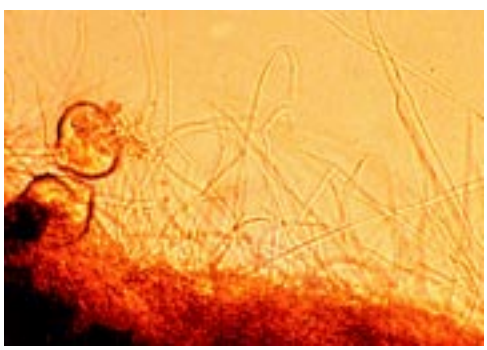

Figure 3-11. Strands of filamentous bacterium *Leucothrix* sp. on heavily infested gills of juvenile *Penaeus monodon*. At left is the protozoan *Zoothamnium* (fresh mount, 200x)

DIAGNOSIS:

Direct microscopic examination of wet mounts of larvae or postlarvae, appendages and gill filaments excised from juvenile or adult shrimp, and of filamentous organisms attached to external surfaces of the cuticle.

PREVENTION AND CONTROL:

- Maintain good water quality with optimum dissolved oxygen and low organic matter levels.
- Apply Cutrine Plus at 0.15 ppm copper in 24 h flowthrough treatments
- Apply 0.5 ppm copper in 4 to 6-h static treatments for PL 2 and older.

Cuticular or Subcuticular Localized Infections

Shell Disease, Brown/Black Spot, Black Rot/Erosion, Blisters, Necrosis of Appendages

CAUSATIVE AGENT:

Shell-degrading bacteria belonging to *Vibrio, Aeromonas, and Pseudomonas* groups

Species affected:

Penaeus monodon, P. merguiensis, P. indicus

GROSS SIGNS:

Figure 3-12a. Shell disease on the carapace of *Penaeus monodon*

Figure 3-12b. Shell disease on the abdominal segment of *Penaeus monodon*

Figure 3-13. *Penaeus monodon* post larvae with necrotic pleopods. Necrotic area appears like cigarette butt

The disease manifests itself as brownish to black, single or multiple, eroded areas on the general body cuticle (Fig. 3-12a, 3-12b), appendages, and gills.

In larval and postlarval stages, the affected appendage shows a cigarette butt-like appearance (Fig. 3-13). Blister containing cyanotic gelatinous fluid may develop on the carapace and abdominal segment. The blister may extend to the underside of the ventro-lateral section of the carapace creating a bulge on the underside.

EFFECTS ON HOST:

Infection usually starts at sites of punctures or injuries caused by the telson or rostrum, in cracks on the abdominal segment from sudden flexure of the shrimp body, or from other damage caused by cannibalism. Another infection site is the cuticle colonized by a large number of bacteria. The bacteria produce extracellular lipases, proteases, and chitinases, which together erode the multiple-layered cuticle, resulting in the development of the disease. The progressive destruction of the cuticle also provides a route of entry for secondary pathogens like fungi or opportunistic bacteria. Such infections may become lethal because of osmotic imbalances, molting problems, secondary fungal infection and a generalized septicemia. The affected shrimp becomes susceptible to cannibalism or dies from stress and energy exhaustion. The disease is associated with trauma to the cuticle (e.g. heavy aeration), conditions that encourage a high number of bacteria in the culture water (e.g. poor hatchery hygiene, high organic loads or contaminated algae) and undefined nutritional and environmental stressors.

DIAGNOSIS:

Diseased penaeids are examined for appearance of multifocal melanized cuticular lesions on the cuticle or the general body surface, the appendages, or the gills. Diagnosis may also be made by bacteriological (isolation, purification and identification) and serological (slide agglutination) methods.

PREVENTION AND CONTROL:

- Maintain good water quality and use nutritionally adequate diets.
- Keep organic load of the water at low levels by removing sediments

which harbor high numbers of bacteria.

- Minimize handling and overcrowding and reduce other forms of stress.
- Avoid injuries to the exoskeleton of the shrimps to prevent the development of primary portals of entry.
- Induce molting, which eliminates the condition, but not when underlying tissues are damaged.

Internal or Systemic Infections

Luminous Bacterial Disease

CAUSATIVE AGENT:

Vibrio harveyi (Fig. 3-14) and *V. splendidus*

SPECIES AFFECTED:

Penaeus monodon, *P. merguiensis*, and *P. indicus*

STAGES AFFECTED:

Eggs, larvae, postlarvae, juveniles and adults

GROSS SIGNS:

Shrimps become weak and opaque-white. Affected shrimps often swim to the pond surface and edges. Heavily infected shrimps in tanks and ponds show a continuous greenish glow when observed in total darkness. When viewed under the microscope, the hemocoel and internal tissues appear densely packed with active bacteria.

EFFECTS ON HOST:

The hepatopancreas is the target organ of infection. Histopathology shows severe inflammation in and around hepatopancreatic tubules of the entire organ. In larger animals, melanized lesions are found in the proximal region of the hepatopancreas. These lesions affect the digestive function of the organ as the necrotic parts become non-functional. Total necrosis and dysfunction lead to death, while partial dysfunction causes slow growth as not all tubules function in digestion, absorption and storage. Systemic infections result in mortality of up to 100%.

Figure 3-14. Agar culture of the luminous bacteria *Vibrio harveyi*. Photo taken in total darkness

DIAGNOSIS:

The disease may be detected by bacteriological (isolation, purification and identification); histological (demonstration of rod-shaped bacteria in tissues of diseased shrimp); and serological [slide agglutination, fluorescent antibody technique (FAT) and enzyme linked immunosorbent assay (ELISA) using specific antibodies] methods.

PREVENTION AND CONTROL:

- Disinfect incoming water and use filtration equipment to prevent entry of luminous bacteria into the hatchery system.
- Use only previously disinfected water during spawning and rearing.
- Wash eggs.

- Siphon out sediments and debris from the tank bottom.
- Disinfect infected stock first before discarding.
- Wash and disinfect hatchery paraphernalia after each larval rearing period.
- Use microbially mature or aged seawater.
- Apply commercially available probiotics to maintain ecological balance within the system.
- Use immunoprophylaxis or vaccination.
- Monitor bacterial population and diversity in the intake and rearing waters of the shrimp pond.
- Apply commercially available probiotics.
- Use low salinity rearing water and reservoirs.
- Practice crop rotation.
- Install greenwater culture system and other system modifications.
- The disease may be prevented by rigorous water management.
- Apply antibiotics and other antibacterial substances only as the last resort.

Non-luminous Vibrios

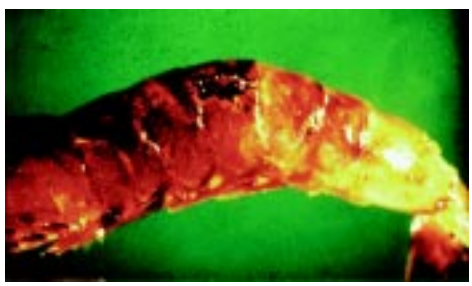

Figure 3-15. *Penaeus monodon* with melanized cuticular lesion on the body surface due to bacterial infection. Whitening of the necrotic muscle at the 6th abdominal segment can also be observed

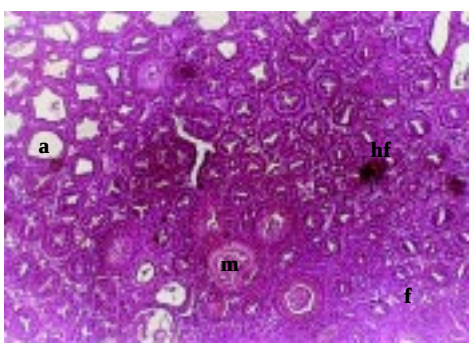

Figure 3-16. Hepatopancreas of *Penaeus monodon* infected with *Vibrio parahaemolyticus*. Note the presence of atrophied tubules (a) and melanized tubules (m). The intertubular spaces are infiltrated with hemocytes (hf) and connective tissues (f). (Hematoxylin and Eosin stain, 100x)

CAUSATIVE AGENT:

Vibrio parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. damsela, V. fluvialis and *V. penaeicida.*

SPECIES AFFECTED:

Penaeus monodon, P. vannamei, P. japonicus

GROSS SIGNS:

Affected shrimp may show erratic or disoriented swimming alternating with periods of lethargy. There is loss of appetite. General signs of severe stress include opaqueness of abdominal muscle (Fig. 3-15), anorexia and expansion of chromatophores.

In larval and early postlarval shrimp, signs include melanization, necrosis of appendage tips and the presence of large numbers of swarming bacteria visible in the hemocoel of moribund or weak shrimp. Due to loss of appetite, fecal strands cannot be observed and gut is empty. Hepatopancreas of affected shrimp showed varying degrees of inflammation, hemocytic infiltration and fibriosis (Fig. 3-16).

EFFECTS ON HOST:

Mortality in some instances is nearly 100% of affected population. Majority of cases of vibriosis is secondary in nature, occurring as a result of other primary conditions, including other infectious diseases, nutritional diseases, extreme stress, wounds, etc.

DIAGNOSIS:

Infection may be detected by bacteriological (isolation, purification and identification), histological (demonstration of rod-shaped bacteria

in tissues of diseased shrimp), and serological (slide agglutination, FAT and ELISA using specific antibodies) methods; and by Polymerase Chain Reaction (PCR).

PREVENTION AND CONTROL:

- Maintain good water quality and use nutritionally adequate diets.
- Minimize handling and overcrowding; reduce effects of other forms of stress.
- Apply commercially available probiotics; use low salinity rearing water and reservoirs; practice crop rotation and install greenwater culture system and other system modifications.
- Perform immunoprophylaxis or vaccination.
- The disease can be treated with rigorous water management.
- Apply antibiotics and antibacterial substances only as a last resort.

SUMMARY

Fish and crustaceans that are not weakened by poor environmental conditions, or by other causes, such as parasitic infestation, nutritional deficiency, handling stress, or chemical intoxication, are more resistant to bacterial infections. This is due to the presence of a large amount of bactericidal substances in the blood, which helps overcome infections. So, the best precaution against the occurrence of bacterial infections is to provide the fish with optimum environmental conditions, adequate amounts of the right kinds of food and avoidance of stress, including overcrowding. Vaccination/ immunization and genetic manipulation (i.e., the development of specific pathogen resistant fry) are also some ways of preventing bacterial diseases. The use of antibiotics should always be an option of the last resort.

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4

CHAPTER FOUR

Fungal diseases

Eduardo M. Leaño

Many diseases of aquatic animals are caused by organisms that are part of the normal biota of their surrounding environment. Fungi and fungal-like organisms (straminipilous organisms) occur in most waters. They are either saprobes that colonize decaying organic matter, or parasites, which attack a great variety of aquatic organisms leading to disease outbreaks. Among the parasitic species, many are known to be important causative agents of aquatic animal diseases. They are generally opportunistic invaders, but once established, are often fatal and difficult to treat. Thus, fungi and straminipilous organisms may be problematic pathogens under stressful conditions in an aquaculture system.

This chapter lists the important fungal diseases of freshwater and marine animals that are commonly encountered in the Philippines and other Asian countries.

WHAT ARE FUNGI?

Fungi constitute a group of heterotrophic organisms, which contain no chlorophyll and are historically compared to plants. They are usually filamentous and multicellular, although some are non-filamentous and unicellular. The filaments known as **hyphae** (sing. hypha) constitute the body of a fungus. These filaments elongate by apical growth (growth is active at hyphal tips), in contrast to intercallary growth of other filamentous organisms. The hyphae are either **septate** (divided by cross walls) or **non-septate** (coenocytic, without cross walls) (Fig. 4-1). They branch successively behind the tips, resulting in a network of hyphae called **mycelium** (pl. mycelia).

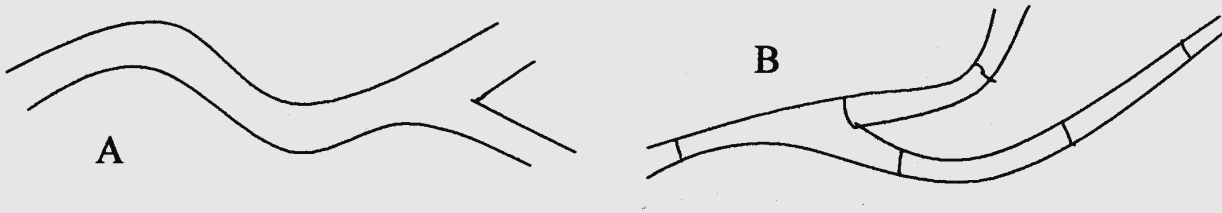

Figure 4-1. Non-septate (A) and septate (B) hyphae

Most parts of the fungal body (also known as **soma** or **thallus**) are potentially capable of growth. A minute fragment from most parts of the organism is able to produce a new growing point, and to start a new individual. In general, fungi reproduce by both asexual and sexual means (Fig. 4-2), producing different kinds of spores as end products. The reproductive structures are usually differentiated from the somatic structures. They occur in a variety of forms, which are usually the basis for classifying different species.

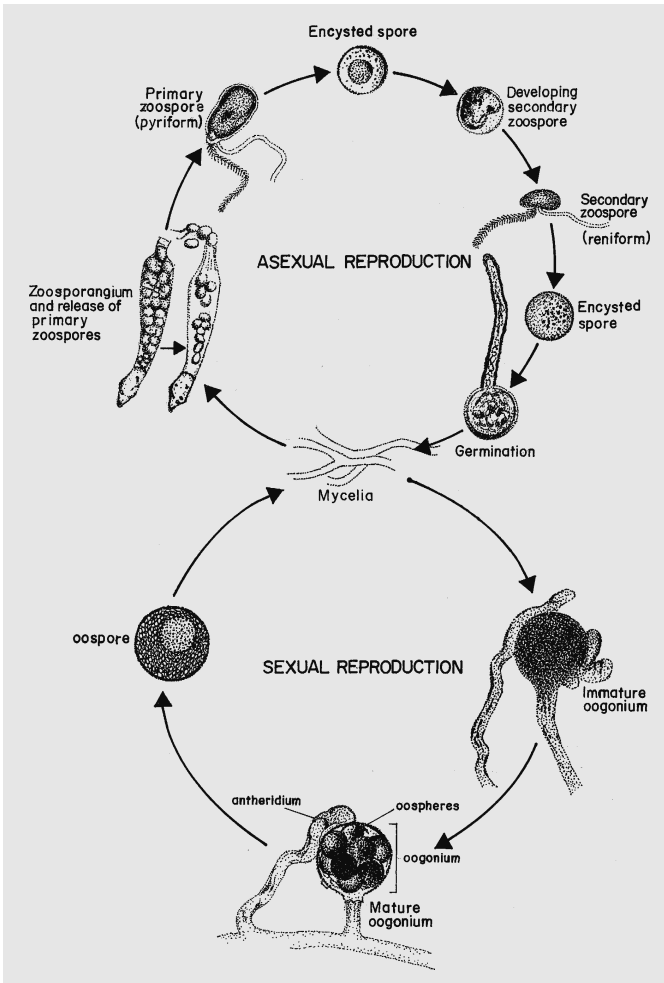

Figure 4-2. Life cycle of *Saprolegnia* (not drawn to scale)

Fungal cell walls are primarily made up of chitin. This characteristic is one of the basis for separating lower fungi (also known as zoosporic fungi) into another kingdom – Kingdom Stramenopila; as the cell wall of this group of organisms is made up primarily of cellulose. Lower fungi are now referred to as stramenopiles or straminipilous organisms. This group of zoosporic organisms causes most fungal diseases of aquatic animals.

As heterotrophs, fungi exhibit absorptive nutrition. They have a remarkable ability to utilize almost any carbon source as food. Many fungi are saprobes (saprotrophs), which obtain their food from dead or decaying organic matter. On the other hand, a considerable number of species live as parasites of plants, animals, and in some cases, even other fungi. Majority of fungi are also capable of living on dead organic materials, as shown by their ability to grow on synthetic media. These organisms are known as **facultative parasites** or **facultative saprobes**. There are some parasitic species, which cannot be cultured in synthetic media, referred to as **obligate parasites** or **biotrophs**. Still others can form mutualistic relationships with either animal or plant host.

Fungi and straminipilous organisms (oomycetes) may be isolated from nearly any organic detritus or biological surfaces. Most are ubiquitous in freshwater, estuarine or coastal marine habitats. For straminipilous organisms, there are two isolation methods commonly used:

a) placing of appropriate substrate on a more or less selective medium supplemented with antibiotics to inhibit bacterial growth; and,

b) baiting water with selective substrate such as pollen, insect exuviae, and cellulose.

Freshwater oomycetes (e.g. *Saprolegnia* and *Achlya*) are readily isolated by baiting pond waters, and from fish with fungal infection. Holocarpic oomycetes such as *Lagenidium callinectes* and *Haliphthoros milfordensis*, which are facultative parasites of eggs and larvae of marine crustaceans, may also be isolated

with a bit more effort and at the appropriate season, when female crabs and shrimps are carrying eggs.

Isolation of pathogenic oomycetes is usually done by inoculating infected tissues into a suitable medium. Using this method, the inoculated medium needs to be observed for fungal growth within a week (sometimes after 24 to 48 h) to facilitate subculture and identification. Isolation using baits may stand for several weeks without being overgrown by contaminants.

Identification of fungal pathogens are usually based on the fruiting bodies (zoosporangia or conidia) produced by the organism, either on the infected tissues or from axenic cultures. Hyphae of straminipilous organisms are usually wide, hyaline, and non-septate, while those of the higher fungi are thin and septate.

MAJOR FUNGAL DISEASES OF FISH

Mycotic infections among freshwater fish species are commonly caused by straminipilous organisms. The pathogens can infect eggs, fry, fingerlings, and adult fish. Stress factors such as mechanical injury after handling, exposure to extreme pH levels, prolonged exposure to low water temperatures, lack of food, and presence of other microbial infections (e.g. bacterial, viral) increase the susceptibility of fish to fungal infections. Infection is normally restricted to superficial tissues and, unless the fish can be treated, the condition is usually lethal. Listed below are fungal diseases of freshwater fish caused by zoosporic stramenopiles.

Saprolegniosis (Saprolegniasis)

CAUSATIVE AGENTS:

Saprolegnia spp., *Achlya* spp., and *Aphanomyces* spp.

SPECIES AFFECTED:

Many freshwater fish (e.g. carps, goldfish)

Figure 4-3. Mycelial filaments of *Saprolegnia* sp. on the gills of red drum with saprolegniasis (fresh mount, 100x)

GROSS SIGNS:

Formations of white cottony growth on fish eggs and on affected tissues of fish. Virtually, any area on the surface of a fish may become infected but it is usually the integuments that are involved. Gills, eyes and olfactory pits may also become infected (Fig. 4-3). The color of the mycelium may also vary from white to brownish, depending on the color of the particles, which get trapped on the mycelium.

EFFECTS ON HOSTS:

Once infection is initiated, it is generally progressive and terminal. Affected fish become increasingly lethargic, tires more easily and becomes less responsive to external stimuli. Loss of equilibrium often occurs shortly before death. Histopathologically, evidence of rapid destruction of epidermis (tissue necrosis) with slight inflammatory response can be observed.

DIAGNOSIS:

Microscopic examination of the cottony growth from the affected tissues will reveal the characteristic hyaline and coenocytic mycelia. Usually, numerous sporangia will be present. If sporangia are present, one can often make a tentative generic identification. However, it must be remembered that other infections (viral, bacterial, protozoan) that are less easy to diagnose might also be present, and that the occurrence of a saprolegnian infection can mask the characteristic signs of these diseases.

PREVENTION AND CONTROL:

Prevention and treatment of saprolegnian infections of fishes and fish eggs have attracted much attention for a long time now. A vast array of chemicals has been tested for effectiveness against these fungi *in vitro*. Common chemicals used as chemotherapeutants include bath treatments of either of the following:

- Zinc-free malachite green (0.1% on wound and rinse; 67 mg/L for 1 min; 0.2 mg/L for 1 h; 0.1 mg/L indefinite). Cautions: persistent tissue levels; mutagenic; teratogenic; treatment can result in gill damage; more toxic at warm temperatures. Malachite green is considered as the most popular antimycotic agent, being inexpensive and highly effective fungicide. This compound also allows a wide margin of error between therapeutic and toxic dosages.
- Sodium chloride (22 g/L for 30 min; 30 g/L for 10 min; 1-3 g/L indefinite). This compound is safe to use and inexpensive.
- Formalin (0.4-0.5 ml/L 30% formaldehyde for 1 h). Inexpensive and popular chemoprophylactic and chemotherapeutic agent.

Epizootic Ulcerative Syndrome (EUS)

CAUSATIVE AGENTS:

Aphanomyces invadans is associated with the disease outbreak together with rhabdovirus and the bacteria *Aeromonas hydrophila* (Lio-Po 1988). Refer to chapters 2 and 3 for details. Other straminipilous organisms that may superinfect lesions include saprobic *Aphanomyces* strains, *Saprolegnia* spp., and *Pythium* spp.

SPECIES AFFECTED:

More than 30 freshwater fish species (e.g. snakeheads, catfish, guorami, goby, etc.)

GROSS SIGNS:

Early signs observed among affected fish are: darker discoloration and loss of appetite; fish floats just below the water surface, or in some species, with the head just breaking the water surface; occasionally, fish may be hyperactive with a very jerky movement. Ulcerative lesions can be observed throughout the body, which may vary from small areas of rosacea occasionally on the side of the jaw or head, to larger, deep, ulcerative lesions found anywhere on the body.

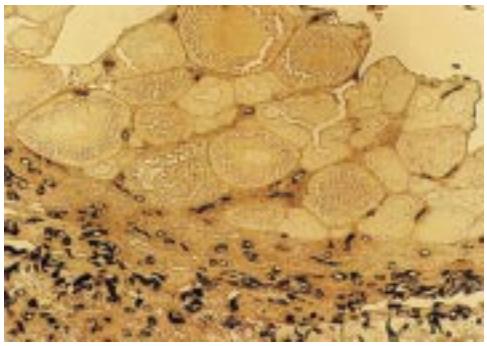

Figure 4-4. Fungal hyphae (black stain) in the connective tissue of the ovary of EUS-infected snakehead, *Ophicephalus striatus*. (Gomori methenamine silver stain, 100x)

EFFECTS ON HOSTS:

Fish affected by the disease become lethargic (inactive or comatose in later stages). Advanced stage of the disease often results in exposed head and bone tissues, visceral organs, and vertebral column. Total erosion of the tail is also common. There is severe hydration problem. Histopathologically, massive infiltration of the muscle tissues by the fungus (Fig. 4-4) accompanied by severe tissue necrosis and minimal inflammatory response can be observed. Fungal hyphae may reach the cranium, kidney, and spinal cord.

DIAGNOSIS:

Outbreaks occur at certain times of the year, normally after flooding followed by cool weather (usually from December until February). Presence of mycotic granulomas which can spread throughout the lesions and also affect some internal organs. Isolation of *A. invadans* from internal tissues.

PREVENTION AND CONTROL:

Since EUS mostly occurs among wild fish stocks, it can be very difficult to control outbreaks within a local area. Therefore, where EUS is not endemic, the most effective means of control would be to prevent entry of any infected fish into the area.

For areas where EUS is presently considered endemic, prevention program should include:

- Eradication of the causative agent (e.g. fungi) by removal of all fish from ponds, reservoirs and water channels prior to restocking; drying-out and liming of ponds; and disinfection of contaminated equipment.
- Once the causative agent has been eradicated from an affected site, reintroduction should be prevented.
- Proper management by reducing stocking densities when EUS prevalence is high in adjacent wild fish populations.
- Farming of EUS-resistant fish species (e.g. tilapia) would also be effective in preventing the occurrence of the disease.

Potentially useful treatments for the causative fungus include:

- 5 ppm Coptrol (a chelated copper compound);
- 0.1 mg/L malachite green.

Branchiomycosis (Gill Rot)

CAUSATIVE AGENTS:

Branchiomyces spp.

SPECIES AFFECTED:

Carps, goldfish, eels

GROSS SIGNS:

Gills become pale with brownish areas due to hemorrhage and thrombosis, or

grayish as a result of ischemia. Necrotic areas might slough-off at a later stage becoming a focus for saprolegnian infections.

EFFECTS ON HOSTS:

Fungal hyphae in the gills obstruct the circulation of the blood. Necrosis and proliferation of lamellar epithelial cells and lamellar fusions may be observed. The disease can appear suddenly and often has a rapid course with losses as high as 30-50% occurring in 2-4 days. Death is due to anoxia.

DIAGNOSIS:

Microscopic examination will reveal the branched and coenocytic mycelia of the pathogen within the affected gill tissues.

PREVENTION AND CONTROL:

Various chemicals have been used to treat branchiomycosis, which include:

- malachite green (0.3 mg/L for 24 h)
- benzalkonium chloride (1-4 ppm active ingredient for 1 h)
- copper sulfate (100 ppm for 10-30 min)
- sodium chloride (3-5%)

If an outbreak occurs, feeding of the fish should be stopped and dead fish should be removed from the ponds and buried in a lime pit. To help prevent further outbreaks, the pond should be drained, dried-out and disinfected with quicklime.

Ichthyophoniasis (Ichthyosporidiosis)

CAUSATIVE AGENT:

Ichthyophonus sp. (= *Ichthyosporidium* sp.)

SPECIES AFFECTED:

Groupers, trouts, flounders, herrings and cods

GROSS SIGNS:

External manifestation of the disease varies from species to species, while some affected fish don't show any external sign. Erratic swimming behavior and swelling of the abdomen are sometimes observed among affected fish. Internal organs (spleen, liver and kidney) become swollen with numerous whitish nodules up to 2 mm diameter. Nodules can also be observed in the muscle tissues of some affected fish.

EFFECTS ON HOST:

Infected fish lose their appetite and become lean and anemic.

DIAGNOSIS

The nodules formed in internal organs or muscle will show different stages of the pathogen (early cyst stage, developed cysts, fungal hyphae) when observed under the microscope. These are surrounded by the hosts' connective tissues forming lumpy granulomas which are typical of a fungus-infected tissue.

PREVENTION AND CONTROL:

There is no known treatment of ichthyophoniasis. Cultured fish become infected when they are fed raw trash fish contaminated with the pathogen. Therefore, contaminated trash fish should be carefully avoided to prevent outbreaks.

MAJOR FUNGAL DISEASES OF CRUSTACEANS

Most marine fungal diseases are encountered among crustaceans, and rarely among fish species. Similar to freshwater condition, most of these diseases are caused by straminipilous organisms, and occasionally by other groups of fungi (e.g. mitosporic and ascomycetes). The eggs and different larval stages are most commonly affected. Listed below are some fungal diseases of cultured shrimps and crabs.

Larval Mycosis

CAUSATIVE AGENTS:

Lagenidium spp., *Sirolpidium* spp., *Haliphthoros* spp.

SPECIES AFFECTED:

All *Penaeus* species, crabs (e.g. *Scylla serrata*)

GROSS SIGNS:

Sudden onset of mortalities in larval stages of shrimps and crabs. Crab eggs are also susceptible for mycotic infection. The commonly affected larval stages among shrimp species are the protozoeal and mysis stages.

EFFECTS OF HOSTS:

Progressive systemic mycosis that is accompanied by little or no host inflammatory response can be observed. Infection is apparently lethal, accumulating mortality of 20-100% within 48-72 h after onset of infection.

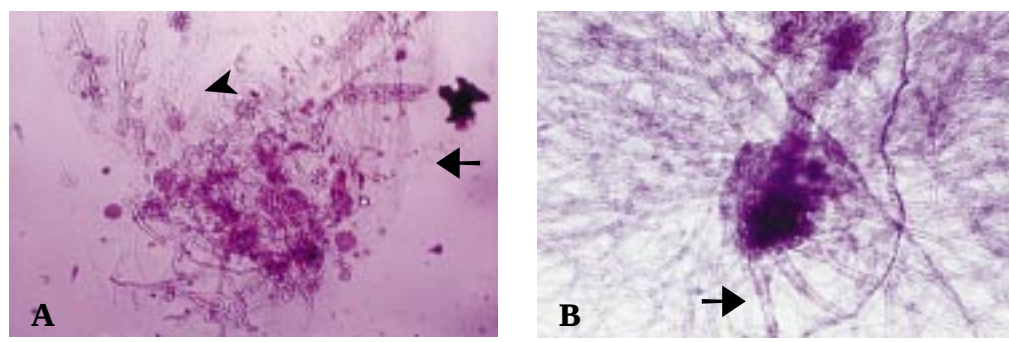

Figure 4-5. *Lagenidium* infection in crustacean larvae. A – Larva of *Penaeus monodon* heavily infested with the fungus (note the mycelia [arrows] completely replacing the body tissues of the larva; and the vesicles [arrowheads] ready to release the zoospores). B – Infected *Scylla serrata* larva in brain-heart-infusion (BHI) broth after 2 days (fresh mount, 40x)

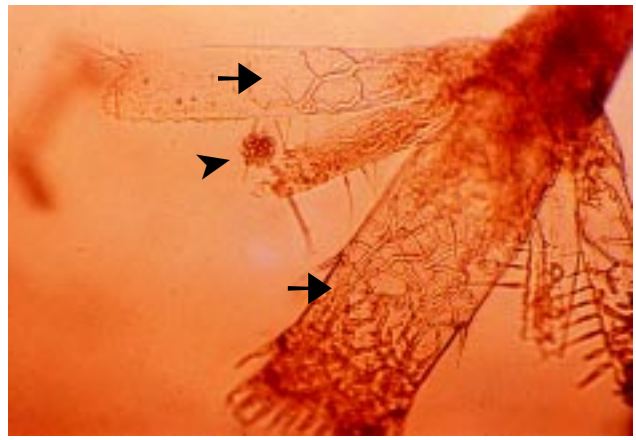

Figure 4-6. Filaments of *Lagenidium* in the tail of *Penaeus monodon* larva (arrows). Zoospores in a vesicle (arrowhead) are about to be released by the fungus (fresh mount, 40x)

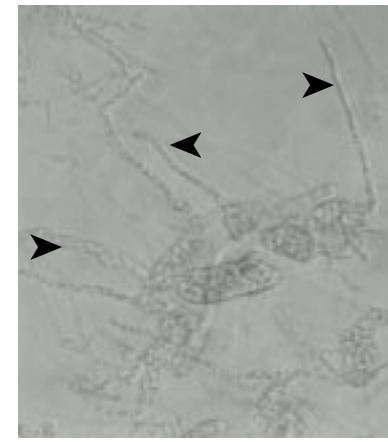

Figure 4-7. Zoosporangial development of *Haliphthoros* sp. by hyphal fragmentation (arrowheads = discharge tubes) (fresh mount, 200x)

DIAGNOSIS:

Microscopic examination of affected larvae will reveal extensive, non-septate, highly branched fungal mycelia throughout the body and appendages (Fig. 4-5). Specialized hyphae or discharge tubes, with or without terminal vesicles, may be present, and could be the basis for identification of the causative agent. Motile zoospores may be observed being released from the discharge tubes in the case of some species. Classification of the type of organism causing particular epizootic of larval mycosis is dependent upon the microscopic examination of sporogenesis as follows:

Lagenidium – zoospores are released from terminal vesicle (Fig. 4-6).

Sirolpidium and *Haliphthoros* – absence of terminal vesicles; zoospores are released through discharge tubes formed by the zoosporangia (Fig. 4-7).

PREVENTION AND CONTROL:

Disinfection of contaminated larval rearing tanks and chlorination and/or filtration of the incoming water can prevent outbreaks. Different antimycotic compounds have been tested *in vitro.* Recommended chemicals for therapeutic and prophylactic treatments include the following:

- 0.2 ppm Treflan
- 1-10 ppm formalin
- egg disinfection with 20 ppm detergent followed by thorough rinsing before hatching

Black Gill Disease (Fusarium Disease)

CAUSATIVE AGENT:

Fusarium solani

SPECIES AFFECTED:

All *Penaeus* species

Figure 4-8. Canoe-shaped macroconidia of *Fusarium* sp. (fresh mount, 400x)

GROSS SIGNS:

Appearance of "black spots" that preceded mortalities in juvenile shrimps grown in ponds.

EFFECTS ON HOSTS:

Infection usually starts on damaged tissues such as wounds, gills damaged from chemical treatments or pollutants, and lesions resulting from other disease processes. Once infection is established, it is usually progressive with 30% remission rate. Lesions may also serve as a route of entry for other opportunistic pathogens.

DIAGNOSIS:

Microscopic examination of wet mounts of infected tissues will reveal the presence of canoe-shaped macroconidia (Fig. 4-8). *Fusarium* spp. are ubiquitous soil fungi. Infection may begin at different loci and spread slowly. *Fusarium solani* is an opportunistic pathogen of penaeids and are capable of establishing infection in shrimps compromised by other stresses or overcrowding.

PREVENTION AND CONTROL:

Preventive measures include the elimination of sources of *Fusarium* conidiophores and destruction of infected individuals. Several fungicides show promise *in vitro* but none proved to be effective in actual field trials.

Aflatoxicosis (Red Disease)

CAUSATIVE AGENT:

Aflatoxin produced by *Aspergillus flavus* and other *Aspergillus* spp. (Fig. 4-9) which are common contaminants of not-properly stored or expired feeds.

SPECIES AFFECTED:

Penaeus monodon, other *Penaeus* spp.

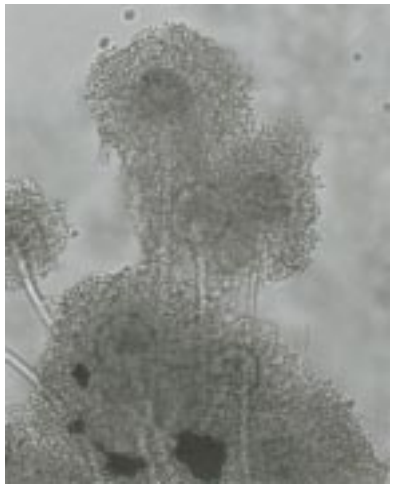

Figure 4-9. Mass of sporangia of *Aspergillus* sp. on contaminated feed particles (fresh mount, 200x)

GROSS SIGNS:

Yellowish, and eventually reddish discoloration of the shrimp body and appendages can be observed among pond-cultured shrimp juveniles. Affected animals become lethargic with weak swimming activity near pond dikes. Soft shelling can also be observed.

DIAGNOSIS:

Affected shrimps will not survive for more than 30 seconds when collected from the feeding trays. There will also be loss of appetite. Confirmation is by chemical analysis for the presence of aflatoxin in the suspected feed/ingredient.

Additional information on aflatoxicosis in shrimp can be found in chapter 7.

EFFECTS ON HOSTS:

Histopathologically, necrosis in the tubule epithelium that proceeds from proximal portion of the tubules to peripheral tubule tips in the hepatopancreas can be observed. Growth will be retarded.

PREVENTION AND CONTROL:

Do not use moldy feeds. Feeds should be properly stored (for not more than 6 months) in dry and well-ventilated areas to prevent, or at least minimize growth of fungal contaminants.

SUMMARY

Over the past 20 years, aquatic animal mycopathogens have become the focus of considerable research. The many known occurrences of fungal diseases in wild populations and the documented devastating disease outbreaks indicate that fungal and fungal-like pathogens are important in nature. Fungal diseases can act as major limitations on natural and cultured populations of aquatic animals. However, knowledge on fungal diseases is rudimentary consisting primarily of the identification and pathology of etiological agents. Detection of fungal infections relies only on the observation of gross pathology, histological examinations, and standard mycological isolation and identification procedures. As a result, there are some cases where the implicated fungal pathogen cannot be demonstrated as the primary cause of a particular disease. In such cases, the fungal pathogen is usually regarded as secondary invader.

Continued research in basic mycology is still an essential resource for fish pathologists in diagnosing diseases caused by fungi. Although fungi reportedly affect very few species, fungal diseases, if not properly controlled or prevented, can still pose a threat to the aquaculture industry.

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5

CHAPTER FIVE

Parasitic diseases and pests

Erlinda R. Cruz-Lacierda

Aquaculture environments that are suitable for growth and reproduction of cultured animals are also hospitable to potential disease agents such as parasites. It is no wonder then that fish mortalities and abnormalities associated with parasites as disease agents are well documented, indicating their importance in aquaculture.

The study of parasites involves an understanding of certain existing relationships in a particular population. **Symbiosis** or "living together" is a relationship that benefits one or both parties. In **commensalism**, no party is harmed and both could live without the other. **Mutualism** is a relationship where both parties benefit from each other, and neither could live without the other. **Parasitism** is a one-way relationship in which one party (the parasite) depends upon, and benefits from, the other partner (the host), biochemically and physiologically.

Parasites live in a variety of environments. Those that live on the external surfaces (skin, fins, gills) of the host are called ectoparasites, while those found in the internal organs are called endoparasites.

This chapter deals with parasitic animals of significance to aquaculture because of their harmful effects on fish and crustaceans. It also discusses the various methods in diagnosing diseases caused by parasites, disease-prevention and control.

COMMON FISH DISEASES CAUSED BY PARASITES

Protozoan Infestations

Protozoans are unicellular, microscopic organisms with specialized structures for locomotion, food gathering, attachment, and protection. They can multiply on or within their hosts.

Ciliates have short, fine cytoplasmic outgrowths called cilia as the locomotory organelle. They are either attached or motile. Ciliates are mainly ectoparasitic.

CAUSATIVE AGENTS:

Ichthyophthirius multifiliis (50-1000 µm diameter) in freshwater (Fig. 5-1a)
Cryptocaryon irritans (60-450 µm diameter) in marine and brackishwater

The disease is known as Ichthyophthiriasis ("Ich") or White Spot Disease

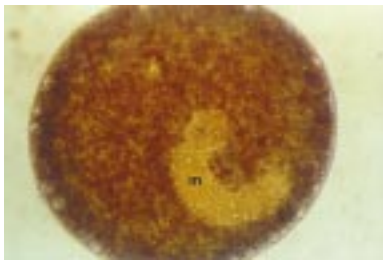

Figure 5-1a. *Ichthyophthirius multifiliis*, mature trophont, from skin of catfish *(Clarias macrocephalus)*. m, macronucleus (400x)

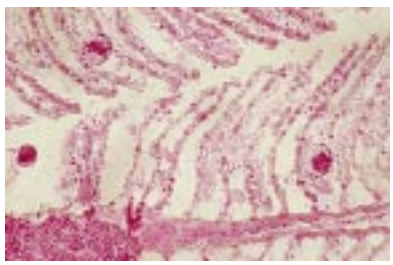

Figure 5-1b. *Cryptocaryon* in gills of fish showing epithelial hyperplasia (Hematoxylin and Eosin, 200x)

Figure 5-2a. *Trichodina* (silver nitrate stain, 400x)

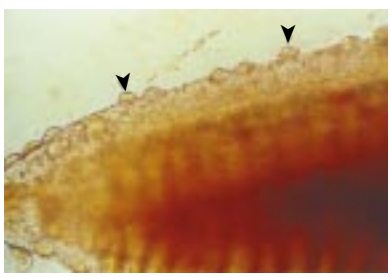

Figure 5-2b. *Trichodina* (arrowheads) on gills of grouper *(Epinephelus coloides)* (fresh mount, 200x)

SPECIES AFFECTED:

Catfish, carp, tilapia, seabass, grouper, snapper

GROSS SIGNS:

The disease is called "white spot" because of the presence of a few to numerous whitish or grayish spots on the skin and gills of affected fish which are actually nests of these parasites. Diseased fish lose their appetite, are lethargic, with dull, opaque or hemorrhagic eyes. Heavily infested fish produce a lot of mucus and they rub their body against the substrate or sides of tanks.

EFFECTS ON HOST:

This disease causes severe epizootic especially in intensive culture systems. The parasite may destroy the skin and gills (Fig. 5-1b). Ulcers may develop in the skin of heavily infested fish and may be the sites of secondary bacterial or fungal infection. Occurrence of this parasite is usually associated with a drop in temperature to 28°C.

DIAGNOSIS:

Encysted (0.10-0.35 mm) organisms appear as white spots on the surface of fish and can be seen by the naked eye. Microscopic examination of mucus from the body surface and gill filaments reveals round or oval parasites, propelled by cilia and possessing a horseshoe-shaped macronucleus in the case of *Ichthyophthirius*.

PREVENTION AND CONTROL:

For "Ich"

- Increase water temperature to 30°C for 6 h daily for 3-5 d
- 0.05% salt solution
- 100 ppm formalin for 1 h for 2-3 d
- 25 ppm formalin + 0.1 ppm malachite green
- Transfer infected stock in dry, parasite-free tanks for 2-3 times at 3 d interval

For *Cryptocaryon*

- 0.5 ppm $CuSO_4$ and 25 ppm formalin for 5-7 d, then transfer to dry, parasite-free tanks for 2 times at 3 d interval.

CAUSATIVE AGENTS:

Trichodina (45-78 µm diameter) (Fig. 5-2a), *Trichodinella* (24-37 µm diameter), *Tripartiella* (up to 40 µm diameter)

SPECIES AFFECTED:

Carp, tilapia, milkfish, seabass, mullet, siganid, grouper, snapper

GROSS SIGNS:

The parasites are attached mainly on the gills (Fig. 5-2b) and skin of the host. Affected fish appear weak with excessive mucus production and with frayed fins.

EFFECTS ON HOST:

Excessive numbers of the parasite on the skin and gills of infested fish may interfere with respiration. High mortality was observed among young fish. The adhesive disc can cause direct damage to the branchial epithelium resulting in gill lesions.

DIAGNOSIS:

Microscopic examination of wet mounts of gill filaments and scrapings from skin show saucer-shaped organisms, surrounded by cilia around its perimeter.

PREVENTION AND CONTROL:

- 2-3% salt solution for 2-5 min for 3-4 d (carp fry)
- 100% freshwater bath for 1 h for 3 d
- 100 ppm formalin + 10 ppm Acriflavin for 1 h for 3 d

CAUSATIVE AGENT:

Brooklynella (36-86 x 32-50 µm)

SPECIES AFFECTED:

Grouper, seabass, snapper

GROSS SIGNS:

The parasite attaches to the skin and gills of fish. Affected fish rub body against objects causing extensive skin damage and subcutaneous hemorrhage.

EFFECTS ON HOST:

May result to respiratory difficulties; may develop secondary bacterial infection

DIAGNOSIS:

Microscopic examination of mucus from body surface of affected fish and gill filaments show bean-shaped protozoans with long parallel lines of cilia that beat in waves.

PREVENTION AND CONTROL:

- 100% freshwater bath for 1 h for 3 days
- 100 ppm formalin for 1 h for 2-3 days

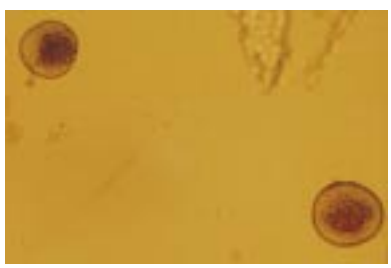

Figure 5-3a. Trophont of *Amyloodinium* fixed in Bouin's solution (200x)

Flagellates have one or more long, hair-like structures called flagella used as a locomotory organelle. They occur on the skin, gills, intestinal organs, and blood of fish.

CAUSATIVE AGENT:

Amyloodinium ocellatum (150-350 x 15-70 µm) (Fig. 5-3a, 5-3b)

SPECIES AFFECTED:

Mullet, siganid, grouper

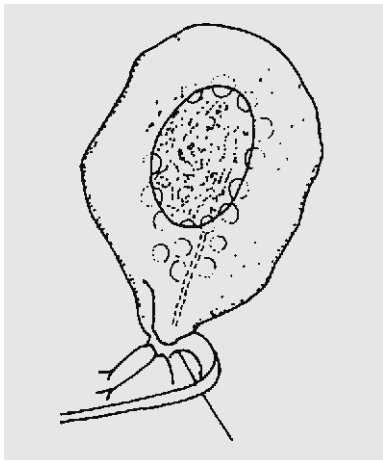

Figure 5-3b. *Amyloodinium ocellatum*, young trophont

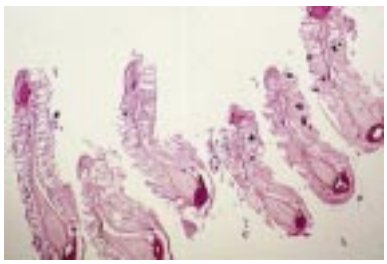

Figure 5-3c. Gill filaments of fish parasitized by *Amyloodinium* showing epithelial hyperplasia and disintegrating inner layer (Hematoxylin and Eosin, 100x)

GROSS SIGNS:

Heavily infested skin may have a dusty appearance ('velvet disease') with excessive mucus production. The parasite also attaches to the gills of affected fish. Fish rub body against objects in tanks. Affected fish exhibit abnormal surface swimming (spasmodic gasping and uncoordinated movements).

EFFECTS ON HOST:

This disease has been reported to cause morbidity and mortality in marine and brackishwater fishes. Heavy infestation can cause death within half a day. Histopathological changes include disintegration of the affected tissues (Fig. 5-3c), severe gill epithelial hyperplasia and reduced or absence of mucus cell.

DIAGNOSIS:

Microscopic examination of gill filaments or skin scrapings will reveal pear or ovoid-shaped trophonts with elongated red stigma near attachment site.

PREVENTION AND CONTROL:

- Use of sand filters; ultraviolet irradiation of rearing water
- Disinfection of culture facilities using lime
- Quarantine of new stocks
- Freshwater bath can cause parasite to drop off the gills
- 0.75 ppm $CuSO_4$ for 5-6 days
- 25 ppm formalin plus 0.1 ppm malachite green for 1 day
- 100-300 ppm formalin, 10 min

CAUSATIVE AGENTS:

Trypanosoma (18-32 µm) (Fig. 5-4a), *Cryptobia* (15 µm) (Fig. 5-4b), *Ichthyobodo* (10-15 µm) (Fig. 5-4c)

SPECIES/TISSUE AFFECTED:

Snakehead, carps, mullet, milkfish (blood)

GROSS SIGNS:

Affected fish have greyish-white film on fins and body surface, with frayed

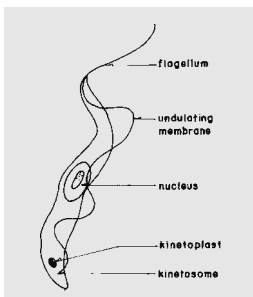

Figure 5-4a. *Trypanosoma*, adult stage

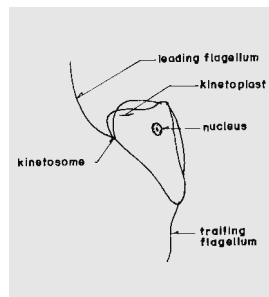

Figure 5-4b. *Cryptobia*

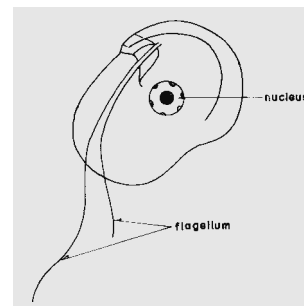

Figure 5-4c. *Ichthyobodo*

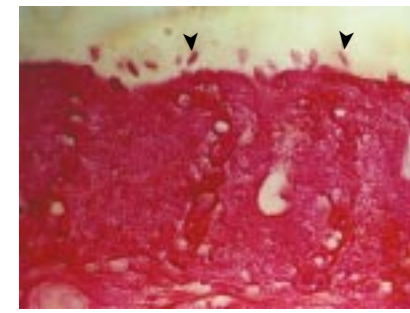

Figure 5-4d. Histological section of gills of snakehead *(Ophicephalus striatus)* with *Ichthyobodo* (arrowheads). (Hematoxylin and Eosin, 400x)

or destroyed fins. Fish rub their body against immersed objects or sides of the tank. *Ichthyobodo* is attached mainly on dorsal fins and gills (Fig. 5-4d) of the host. *Trypanosoma* and *Cryptobia* are parasitic on the blood of fish.

EFFECT ON HOST:

Affected fish show sluggishness, pale gills, and emaciated body. Fish parasitized by blood protozoans are usually anemic.

DIAGNOSIS:

For *Ichthyobodo*, microscopic examination of mucus from body surface and gill filaments. For blood protozoans, blood smears fixed in methanol and stained with Giemsa are examined under high power magnification (100x) of a compound microscope.

PREVENTION AND CONTROL:

- Drying of culture facilities
- Use of filters
- Elimination of the vector (leech) for blood protozoans
- Application of 10 ppt, 15-30 min or 2-5 ppm $KMnO_4$

Myxosporeans – the spore (7-20 µm) is the infective stage, and is composed of 1 to 7-spore shell valves, 1 to 2-sporoplasms and 2 to 7-polar capsules. Myxosporeans are parasitic in organ cavities and tissues of fish (Fig. 5-5).

CAUSATIVE AGENT:

Myxidium, Myxobolus, Henneguya, Kudoa, Myxosoma, Thelohanellus

SPECIES AFFECTED:

Mullet, catfish, eel, carps, climbing perch, snakehead

GROSS SIGNS:

White cysts are formed on skin, gills, muscle, brain, heart, ovaries, or other internal organs of fish. Myxosporean cysts produce thick milky exudate when ruptured.

EFFECT ON HOST:

Heavy gill infections may lead to respiratory dysfunction. Several cysts formed in the muscle may render the fish unmarketable. Heavy infection in internal organs may result to loss of equilibrium, skeletal deformities, and destruction of the host tissue.

DIAGNOSIS:

Microscopic examination of fresh smears of cysts containing many infective spores.

PREVENTION AND CONTROL:

- Isolate and destroy infected fish
- Disinfect rearing facilities with lime

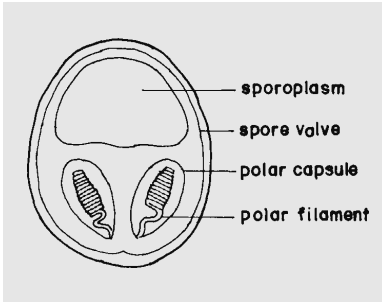

Figure 5-5. Generalized structure of a myxosporean spore

CAUSATIVE AGENT:

Sphaerospora (8.7 x 8.2 µm; with 2 spherical polar capsules)

SPECIES AFFECTED:

Grouper, seabass, marine catfish

GROSS SIGNS:

Affected fish exhibit swollen abdomen, exophthalmia and anemia.

EFFECTS ON HOST:

Spore stages are found in kidney, liver, gall bladder, and blood cells. Infected kidney tubules display severe vacuolation of the epithelium.

DIAGNOSIS:

Microscopic examination of fresh preparations of kidney and blood smears stained with Giemsa.

PREVENTION AND CONTROL:

Ultraviolet treatment of inflow water can control the infective stage, but is usually impractical

Monogenean Infestations

Monogeneans are ectoparasitic flatworms, < 1-5 mm long, with posterior organ of attachment called haptor armed with hooks and/or suckers (Fig. 5-6a)

CAUSATIVE AGENTS:

Gyrodactylus (Fig. 5-6b), *Dactylogyrus* (Fig. 5-6c), *Pseudorhabdosynochus* (Fig. 5-6d), *Benedenia* (Fig. 5-6e)

SPECIES AFFECTED:

Catfish, carp, tilapia, seabass, grouper, snapper

GROSS SIGNS:

Parasite attaches on gills (Fig. 5-6f), fins and body surface of fish. Affected fish have pale skin and gills with increased mucus production, frayed fins, and the cornea may become opaque.

EFFECTS ON HOST:

Heavy infestation may result to hyperplasia of the epithelial cells in the skin. Extensive damage to the gill epithelium may affect normal respiration. Heavy infestations may result in mortality. Conditions of low oxygen levels may increase mortality rates. Often associated with vibriosis.

DIAGNOSIS:

Gross and microscopic examination of gills and body surface of freshly sacrificed fish.

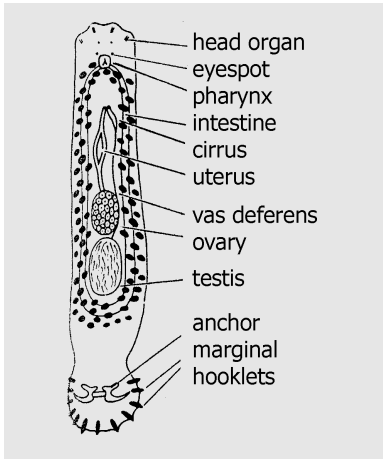

Figure 5-6a. Generalized structure of an adult monogenean

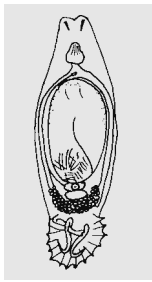

Figure 5-6b. *Gyrodactylus*

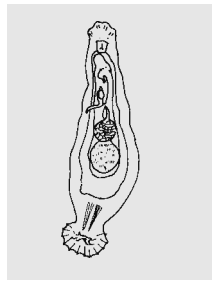

Figure 5-6c. *Dactylogyrus*

Figure 5-6d. *Pseudorhabdosynochus*

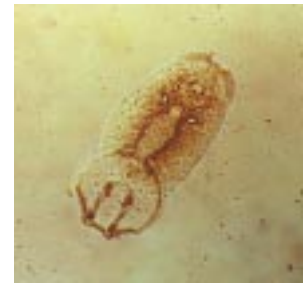

Figure 5-6e. *Benedenia* (fresh mount, 200x)

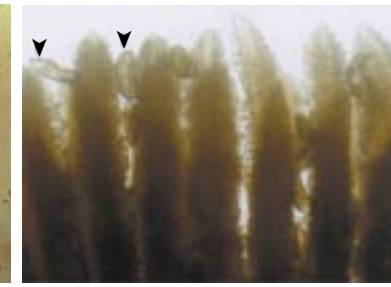

Figure 5-6f. *Pseudorhabdosynochus* (arrows) attached on the gills of grouper *(Epinephelus coioides)* (fresh mount, 100x)

PREVENTION AND CONTROL:

- Maintain optimum stocking density and adequate feeding
- 5% salt solution for 5 minutes
- Freshwater bath for 1 h for 3 days
- 100 ppm formalin for 1 h for 3 days
- 150 ppm hydrogen peroxide for 30 min

Digenean Infestations

Digeneans are endoparasitic flatworms measuring 1-2.6 x 0.2-0.8 mm with 2 sucker-like attachment organs located at the anterior and ventral portions (Fig. 5-7).

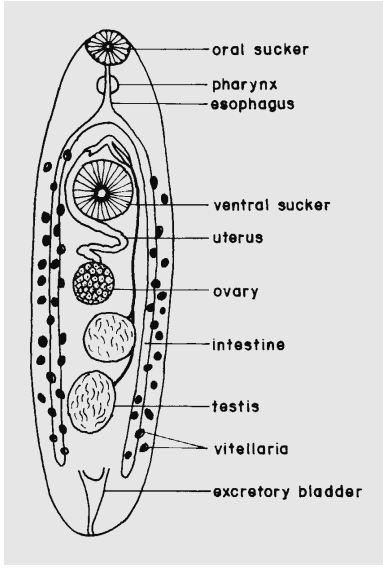

Figure 5-7. Generalized structure of an adult digenean

CAUSATIVE AGENTS:

Bucephalus, Lecithochirium, Pseudometadena, Transversotrema, Stellantchasmus, Haplorchis, Procerovum, Prosorhynchus, Hemiurus

SPECIES AFFECTED:

Bighead carp, grass carp, milkfish, seabass, grouper, siganid, mullet

GROSS SIGNS:

Presence of small, white to yellow or brown to black cysts on the skin, fins, gills, muscle, stomach or intestine. Affected fish have distended abdomen. Growth retardation has been observed in some cases.

EFFECTS ON HOST:

Affects growth and survival or disfigures fish. Disrupts function of vital organs. It may cause mild diarrhea to cardiac and visceral complications in humans (definitive host).

DIAGNOSIS:

Gross and microscopic examinations of the gills, muscle and internal organs for opaque or creamy cysts containing motile metacercariae.

PREVENTION AND CONTROL:

Elimination of intermediate host

Cestode Infestations

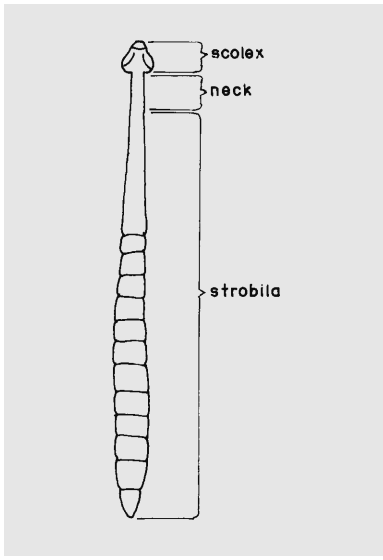

Figure 5-8. Generalized structure of a cestode

Cestodes are endoparasitic tapeworms, body is ribbon-like, segmented or unsegmented, 5-70 mm long and with an anterior attachment organ called scolex armed with hooks or suckers (Fig. 5-8).

CAUSATIVE AGENT:

Botriocephalus

SPECIES AFFECTED:

Carps, catfish, snakehead

GROSS SIGNS:

Affected fish are sluggish, with emaciated body because of non-feeding. This parasite is commonly found in intestine of fish.

EFFECTS ON HOST:

The parasite may induce hemorrhagic enteritis due to destruction of the intestinal epithelium. Adult stage of the parasite interferes with absorptive processes of the intestine and may reduce food intake. Secondary microbial infection is possible. Some fish cestodes are important human parasites.

DIAGNOSIS:

Gross examination of the intestine of host fish.

PREVENTION AND CONTROL:

- Elimination of intermediate hosts
- Disinfection of culture facilities with quicklime to destroy cestode eggs

Nematode Infestations

Nematodes are unsegmented roundworms (Fig. 5-9a, b, c); female, 7-21 x 0.18-0.8 mm; male, 3-9 x 0.1-0.5 mm

CAUSATIVE AGENT:

Spirocamallanus, Raphidascaris, Contracaecum, Echinocephalus

SPECIES AFFECTED:

Siganid, grouper, catfish, snakehead, goby

GROSS SIGNS:

Parasitizes the stomach and intestine of host fish. Affected fish have emaciated, discolored body surface and swollen intestine.

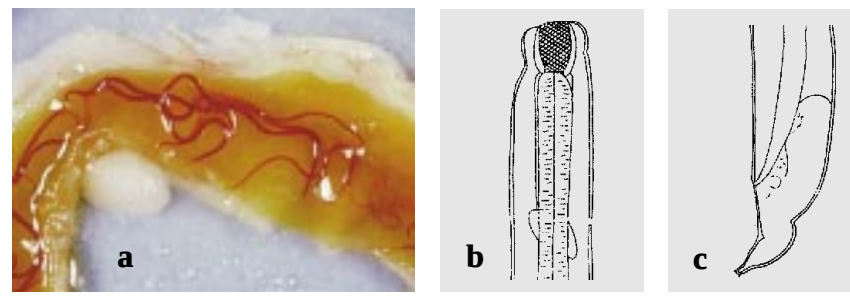

Figure 5-9a. *Spirocamallanus* in intestine of rabbit fish *(Siganus guttatus)*; **9b.** Anterior portion containing the head; **9c.** Female, tail

EFFECTS ON HOST:

May impair feeding, resulting in emaciation, growth retardation, and mild mortalities.

DIAGNOSIS:

Gross examination and dissection of the abdominal area reveals swollen intestine filled with liquid and large worms.

PREVENTION AND CONTROL:

- Elimination of intermediate hosts
- Drying of pond bottom
- Disinfection of culture facilities with quicklime to destroy nematode eggs
- Filtration

Acanthocephalan Infestations

Acanthocephalans are "thorny- or spiny-headed" elongated (10 mm long) cylindrical worms, having an anterior refractile proboscis with hooks (Fig. 5-10).

CAUSATIVE AGENT:

Acanthocephalus, Pallisentis

SPECIES AFFECTED:

Snakehead, catfish, eel, tilapia, milkfish

GROSS SIGNS:

The parasite is attached to intestinal mucosa of the host. Affected fish have darkened, emaciated body.

EFFECTS ON HOST:

The parasite causes necrotic hemorrhagic ulcers in the intestine of the host. Growth retardation and mortality have been reported.

DIAGNOSIS:

Gross examination of intestine reveals elongated and sac-like worms with retractile proboscis armed with spines.

PREVENTION AND CONTROL:

- Disinfect pond with quicklime
- Control of water supply and potential intermediate hosts
- Quarantine new and suspected stocks

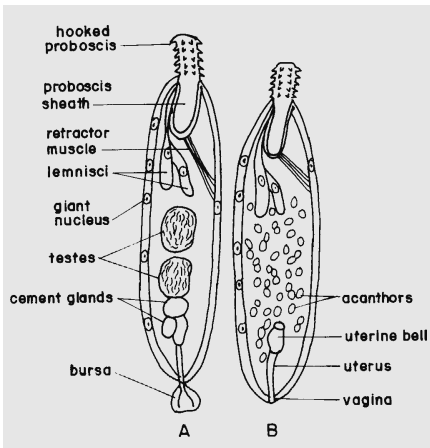

Figure 5-10. Generalized structure of an (a) adult male and (b) adult female acanthocephalans

Crustacean Infestations

Crustacean parasites have segmented bodies covered by shell with jointed appendages.

Argulus (fish louse) infestation – female, 6-6.5 mm; male, 2-3 mm

CAUSATIVE AGENT:

Argulus (Fig. 5-11)

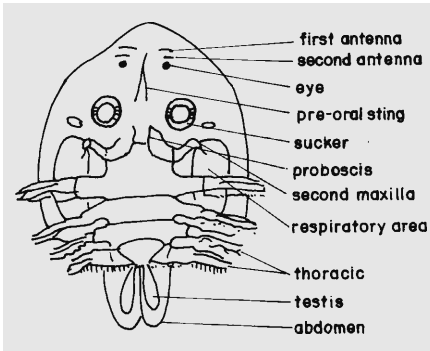

Figure 5-11. Generalized structure of an adult male *Argulus*

SPECIES AFFECTED:

Tilapia, milkfish, mullet, carp, snakehead, catfish

GROSS SIGNS:

Parasite attached to the skin, fins, buccal or opercular mucosa with two conspicuous black spots. Attachment area hemorrhagic or ulcerated.

EFFECT ON HOST:

Heavy infestation may result in mortalities. Wounds may become necrotic and ulcerated, paving the way for secondary bacterial infection.

DIAGNOSIS:

Gross and microscopic examination of host fish.

PREVENTION AND CONTROL:

- Use of filters
- 5 ppm KmnO4, 3-5 min
- 1 ppm Dipterex, 3-6 h

Caligus infestation (sea lice) – female, 3.9-5.1 mm; male, 2.85 mm

CAUSATIVE AGENTS:

Caligus epidemicus, Caligus patulus (Fig. 5-12)

SPECIES AFFECTED:

Milkfish, seabass, mullet, siganid, tilapia, snapper, spotted scat

GROSS SIGNS:

Transparent parasites appear like white patches, but are not permanently attached to the skin, fins, and gills. Infested areas have no scales, and are hemorrhagic or ulcerated.

Figure 5-12. *Caligus* from skin of snapper (fresh mount, 400x)

EFFECTS ON HOST:

Infested areas are hemorrhagic. The parasite can also cause skin ulcers. Mortalities after heavy infestations have been reported.

DIAGNOSIS:

Gross and microscopic examination of scrapings from possible infested area.

PREVENTION AND CONTROL:

- Freshwater bath for 24 h
- 0.25 ppm Neguvon for 12-24 h repeated at intervals of several weeks

Ergasilid infestation

CAUSATIVE AGENT:

Ergasilus (Fig. 5-13); 0.9-1.5 mm long

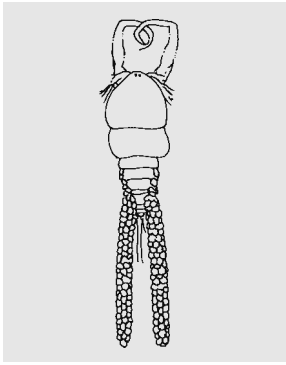

Figure 5-13. Generalized structure of an ergasilid, adult female

SPECIES AFFECTED:

Tilapia, carp, goby, seabass, grouper, mullet

GROSS SIGNS:

Parasite attaches to the gills and body surface of the host. White to dark brown copepods, < 2 mm long, some with 2 white elongated egg sacs are firmly attached to the gills. Affected fish have emaciated body.

EFFECTS ON HOST:

Destruction of the gill filaments resulting to respiratory dysfunction. Infestation of 10-70 parasites per fish can lead to 50% fish loss. Secondary bacterial infection is common.

DIAGNOSIS:

Gross and microscopic examination of host fish.

PREVENTION AND CONTROL:

- 0.15 ppm Bromex bath for 1 week
- 0.25-0.5 ppm Dipterex bath for 24 h

Lernaeid infestation – "anchor worm"; 1.2-1.4 mm long

CAUSATIVE AGENT:

Lernaea (Fig. 5-14)

SPECIES AFFECTED:

Milkfish, carps, tilapia, snakehead, goby

GROSS SIGNS:

Parasites observed protruding from nostrils, skin, bases of fins, gills, buccal cavity. Heavy infestation can result to loss of scale and skin ulcerations.

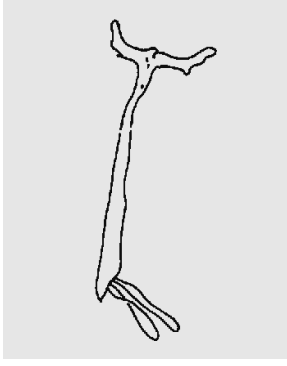

Figure 5-14. Generalized structure of lernaeid, adult female

EFFECTS ON HOST:

The skin and muscle are swollen. Ulcers may develop and result to muscle necrosis. The site of attachment may be a portal of entry for secondary microbial infection. Parasite attachment on the head may cause twisting and deformation of jaws. Affected fish suffer from serious weight loss. Mass mortality is not uncommon.

DIAGNOSIS:

Gross and microscopic examination of host fish. The body shape and attachment organ of the parasite have earned it its name – "anchor worm."

PREVENTION AND CONTROL:

- 3-5% salt solution for control of larval stages
- Adult stages may be eliminated through pond drying and liming

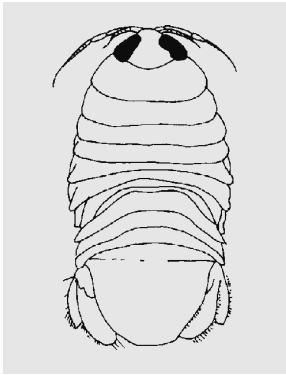

Figure 5-15a. *Alitropus*

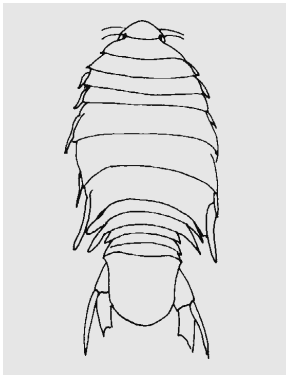

Figure 5-15b. *Nerocila*

Isopod infestation

CAUSATIVE AGENTS:

Alitropus (Fig. 5-15a), *Nerocila* (Fig. 5-15b); 2-3 cm long

SPECIES AFFECTED:

Tilapia, milkfish, seabass, mullet, siganid, grouper, goby

GROSS SIGNS:

The parasite attaches on the skin, mouth and gills of host fish. Clinical signs include reduced opercular movements, loss of appetite, anemia, and slow growth rate.

EFFECTS ON HOST:

The host tissue is destroyed brought about by the pressure of the parasite's body. There is necrosis of the dermis and the gill filaments. Swimming and feeding behavior are affected. Rapid death occurs in 1-2 days particularly in young fish.

DIAGNOSIS:

Gross examination of host fish.

PREVENTION AND CONTROL:

- Mechanical removal
- 200 ppm formalin bath until parasite detaches from the host
- Drying and liming of ponds for several weeks

Marine Leech Infestations

Figure 5-16a. *Zeylanicobdella arugamensis*

Figure 5-16b. Anal fin of *Epinephelus coioides* infested with *Zeylanicobdella arugamensis*

Marine leeches have striated bodies with muscular body wall, with anterior and posterior suckers, usually 8-12 mm long.

CAUSATIVE AGENT:

Zeylanicobdella arugamensis (Fig. 5-16a)

SPECIES AFFECTED:

Grouper, milkfish, tilapia

GROSS SIGNS:

Parasite attaches to the skin, fins (Fig. 5-16b), eyes, nostrils, operculum and inside the mouth. The attachment and feeding sites are hemorrhagic.

EFFECTS ON HOST:

The attachment areas are hemorrhagic. The parasite feeds on the host's blood and may result to anemia. Leeches act as vectors of viruses, bacteria and protozoan blood parasites.

DIAGNOSIS:

Gross examination of the host fish.

PREVENTION AND CONTROL:

- Use of filters
- Mechanical removal
- Complete drying of facilities
- 50-100 ppm formalin bath for 1 h

Mollusc Infestation

CAUSATIVE AGENT:

Glochidia is the larval stage of freshwater bivalve molluscs that may attach to fish. The margins of their shells have sharp teeth (Fig. 5-17).

SPECIES AFFECTED:

Freshwater fish

GROSS SIGNS:

The shell valves are attached to gills and outer surfaces of fish.

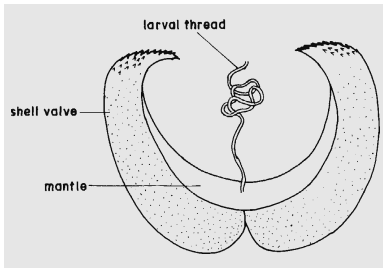

Figure 5-17. Generalized structure of a glochidium

EFFECTS ON HOST:

The gill tissue is destroyed. The respiratory function of the gills during severe infestations is disrupted. Secondary bacterial and fungal infections result when the parasite leaves the host.

Diagnosis:

Gross macroscopic and microscopic examination of the host fish.

PREVENTION AND CONTROL:

- Adequate filtration of intake water to prevent entry of larval glochidium

COMMON CRUSTACEAN DISEASES CAUSED BY PARASITES

Protozoan Infestation

CAUSATIVE AGENTS:

Vorticella (10-150 µm) (Fig. 5-18a), *Zoothamnium* (50-250 µm) (Fig. 5-18b), *Epistylis* (160 µm) (Fig. 5-18c), *Acineta* (35-55 µm) (Fig. 5-18d), *Ephelota* (250 µm) (Fig. 5-18e)

SPECIES AFFECTED:

Shrimps, crabs

GROSS SIGNS:

Heavily infested shrimp have fuzzy mat on gills and body surface.

Figure 5-18a. *Vorticella* (fresh mount, 400x)

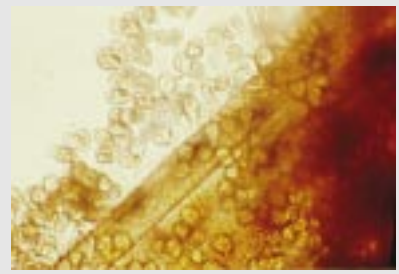

Figure 5-18b. *Zoothamnium* attached to the exoskeleton of shrimp (fresh mount, 200x)

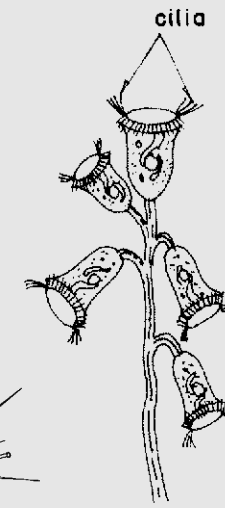

Figure 5-18c. *Epistylis*

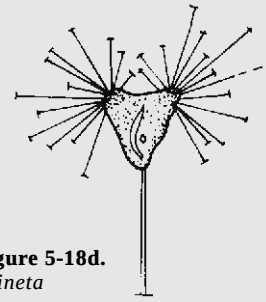

Figure 5-18d. *Acineta*

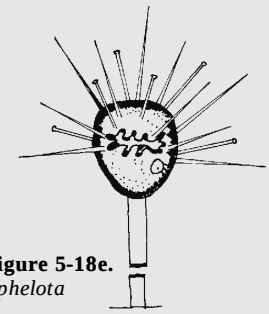

Figure 5-18e. *Ephelota*

EFFECTS ON HOST:

The parasites may cause respiratory and locomotory difficulties when present in large numbers. Heavy infestation may result in mortalities, particularly at low oxygen levels.

DIAGNOSIS:

Microscopic examination of wet mounts of shell and gill scrapings.

PREVENTION AND CONTROL:

- Removal of organic detritus
- Rigid sanitary control of rearing water
- For adult shrimps, 50-100 ppm formalin, 30 min (for *Zoothamnium*) or 30 ppm formalin (for *Epistylis*)

Sporozoans

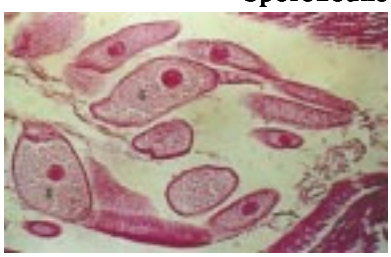

Figure 5-19. Histological section of gut of *Penaeus monodon* juvenile with gregarines (G). (Hematoxylin and Eosin, 400x)

Sporozoans produce simple resistant spores with a special apical complex used in the invasion of the host cell. They can occur in the intestinal organs, muscle tissue and skin of fish.

CAUSATIVE AGENT:

Gregarines (3.5-4 x 8-16 µm) (Fig. 5-19)

SPECIES AFFECTED:

Penaeid shrimps

Gross signs:

Gregarines may be detected in the digestive tract microscopically.

EFFECTS ON HOST:

Large numbers of the parasite attached to filter apparatus of shrimp may possibly interfere with filtration of particles moving towards hepatopancreatic ducts or passing through the stomach. There is a considerable growth retardation. Infection rate in pond-grown shrimps was reported to reach 94%.

DIAGNOSIS:

Microscopic or histological examination of the digestive tract of the host.

PREVENTION AND CONTROL:

- In the hatchery, filter or chlorinate seawater used for rearing
- In grow-out ponds, eliminate the molluscan intermediate host

Microsporeans

These are intracellular parasites with unicellular spores (3-10 µm) containing sporoplasm and coiled polar filament (Fig. 5-20a).

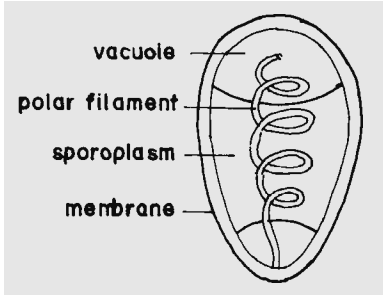

Figure 5-20a. Generalized structure of a microsporidian spore

Figure 5-20b. *Penaeus monodon* with white ovary disease

Figure 5-20c. *Penaeus merguiensis* with microsporeans on the abdominal muscle

CAUSATIVE AGENTS:

Nosema (Ameson), Agmasoma (Thelohania), Pleistophora, Glugea, Ichthyosporidium

SPECIES AFFECTED:

Penaeid shrimps

GROSS SIGNS:

Affected hosts are weakened and easily stressed. Infected areas (cephalothorax, abdominal muscle, ovary) turn opaque white because of the presence of spores and other stages of the parasite, thus the term “cotton” or “milk” shrimp or “white ovary” disease (Fig. 5-20b, 5-20c). Infection may result in sterility of spawners with white ovaries.

EFFECT ON HOST:

Penaeids with spores in the ovaries become sterile. In crabs, microsporidians cause lysis of muscle tissues and increase vulnerability to stress.

DIAGNOSIS:

Microscopic examination of fresh squashes of Giemsa-stained smears from infected areas will reveal spores. Histological sections also provide positive identification.

PREVENTION AND CONTROL:

- Isolate and destroy infected individuals
- Avoid contact of infected broodstock with offspring
- Disinfect culture systems with chlorine or iodine

LIFE CYCLE PATTERNS OF FISH PARASITES

Parasites may have a **direct life cycle**, that is, only one host is needed to complete the parasite's life cycle. Parasites may also have an **indirect life cycle** or they utilize more than one host to complete its life cycle. An **"intermediate host"** is one where the larval stages of the parasite usually develop while the **"final host"** is where the adult stage develops. The final host often feed on the intermediate host. A parasite may also stay in another host, a **"carrier or paratenic" host**, but does not develop in this host. Some parasites are host-specific. This means that they can parasitize only one or a limited number of host species. Parasites that are tissue/ organ-specific parasitize only a particular tissue or organ. An understanding of a parasite's life cycle patterns is useful in disease prevention, since the parasite may be eliminated at the weakest point of its life cycle.

Most protozoan have a direct life cycle. At the infective stages, they are released into the water to reinfect the same host or spread throughout the fish population (e.g. *Ichthyophthirius*) (Fig. 5-21). The blood parasites, e.g. *Cryptobia*, involve the leech, *Piscicola*, as the intermediate host, and are transmitted when the leech takes its blood meal.

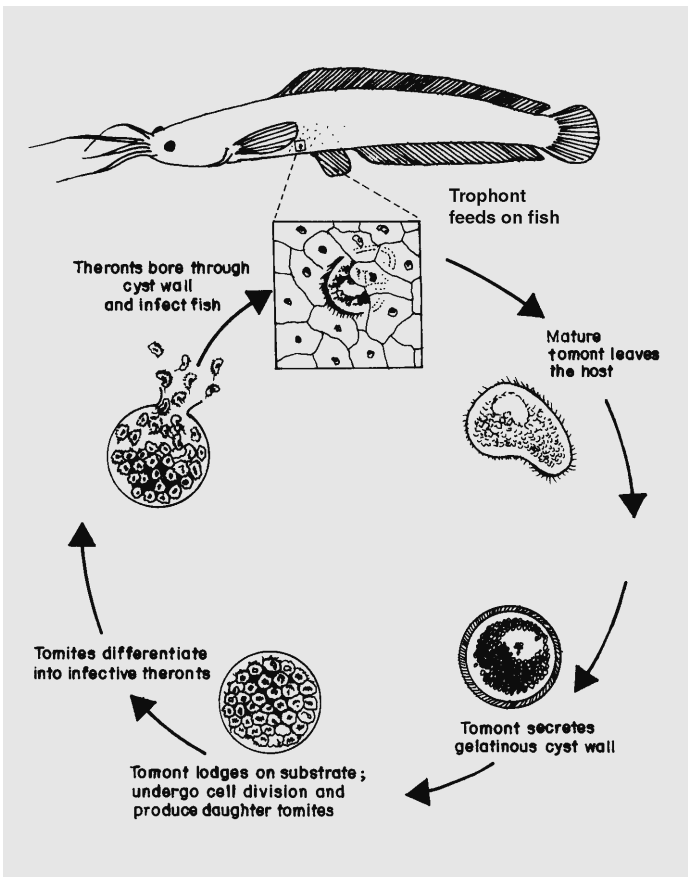

Figure 5-21. Life cycle of *Ichthyophthirius multifiliis*

Monogeneans have a direct life cycle. The free-swimming ciliated larvae **(oncomiracidia)** infect the host within a few hours. The parasites migrate to the final site of attachment and develop into adults (Fig. 5-22). An exception is the gyrodactylid, which bears live young (viviparous) instead of laying eggs. In this case, transfer takes place among fish through physical contact.

Digenean trematodes have an indirect life cycle. Adult parasites lay eggs (oviparous) and hatch as free-swimming larval stage **(miracidium)**. They can survive only for a few hours during which it must find and infect the first intermediate host, often a gastropod or a bivalve mollusc. **Cercariae** develop and encyst into **metacercariae** in a different intermediate host that is eaten by the final host where the adults develop (Fig. 5-23).

Cestodes are also oviparous and require one or more intermediate hosts. Eggs are passed in the feces and may or may not hatch in the water to release a free-swimming larva. The parasite develops further through various stages until finally turning into a **procercoid,** which can infect a fish host.

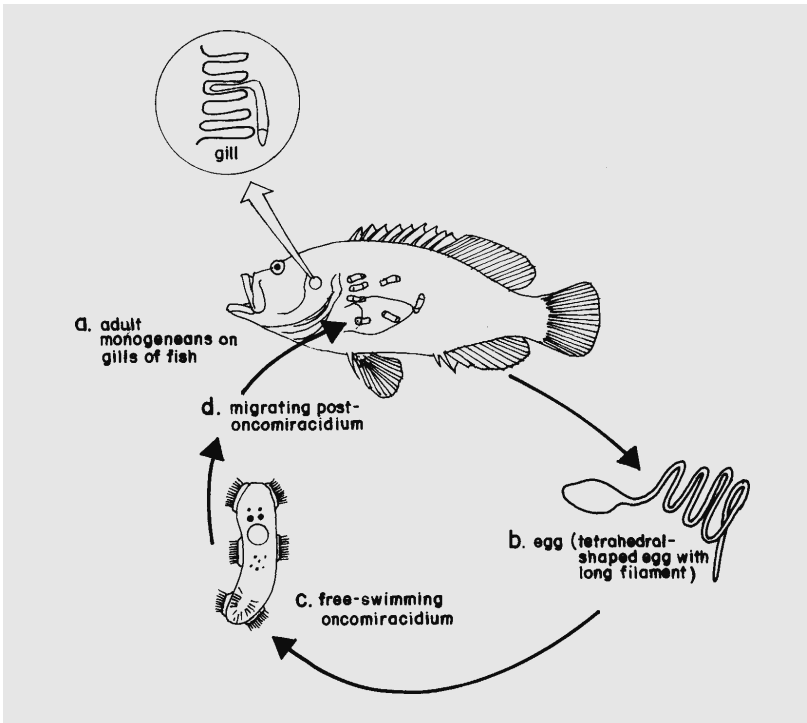

Figure 5-22. Life cycle of monogenean

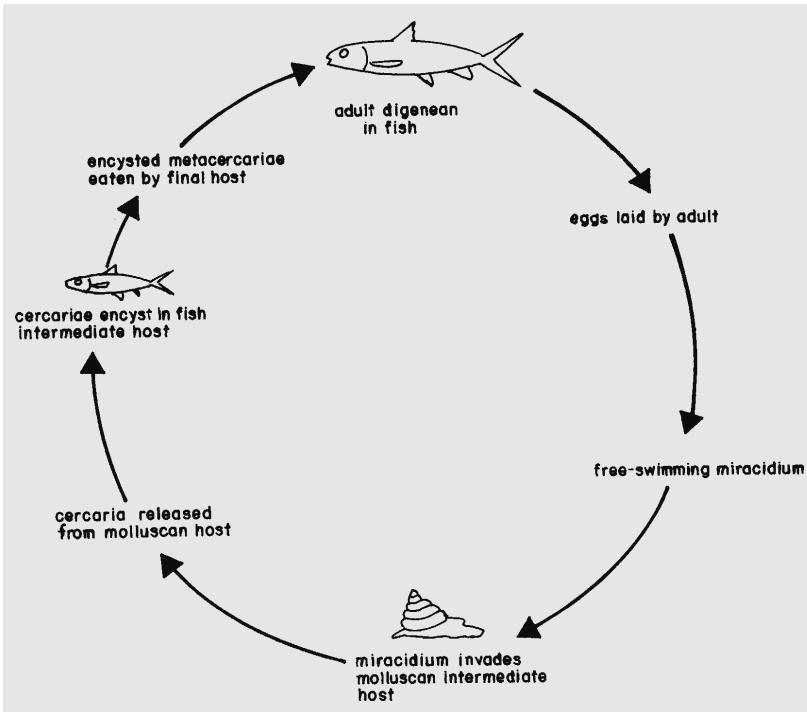

Figure 5-23. Life cycle of digenean

Nematodes are mostly oviparous. The intermediate host is usually an arthropod. The parasite may encyst in viscera and musculature of intermediate or paratenic hosts (Fig. 5-24). The females of orders Camallanoidea and Dracunculoidae are viviparous and release directly into the water.

Acanthocephalans also require an invertebrate host, usually an arthropod, to complete its life cycle.

The freshwater bivalve molluscs of the family Unionidae produce larvae *(glochidia)* which undergo an obligatory phase in the gills, fins or skin of the fish host. The parasitic phase may last for several months. The host tissue surround the parasite until it is shed from the fish, and grows into a free-living adult mollusc.

The mature female ergasilids are usually parasitic, while males are not. In caligids and lernaeids, some or all of the larval copepodid stages may be parasitic on an intermediate fish host or an individual of the final host species (Fig. 5-25). In other species, the males may be hyperparasitic on females.

The parasitic isopods utilize an intermediate host, e.g., a copepod, in their life cycle. In grass shrimp, for example, the female isopods may be found in the gill chamber with the dwarf male among its pleopods. The tiny male fertilizes the ova and the resulting larva swims toward the light, attaches to the copepod intermediate host and develops rapidly into another larval stage. The second stage larva molts and develops further into the larva (cryptoniscus) infective for grass shrimps. Isopoda belonging to the “cyomothoid” type attach to fish early in life and start as a male before developing into a female. Male-stage specimens cannot develop further in the presence of a ma-

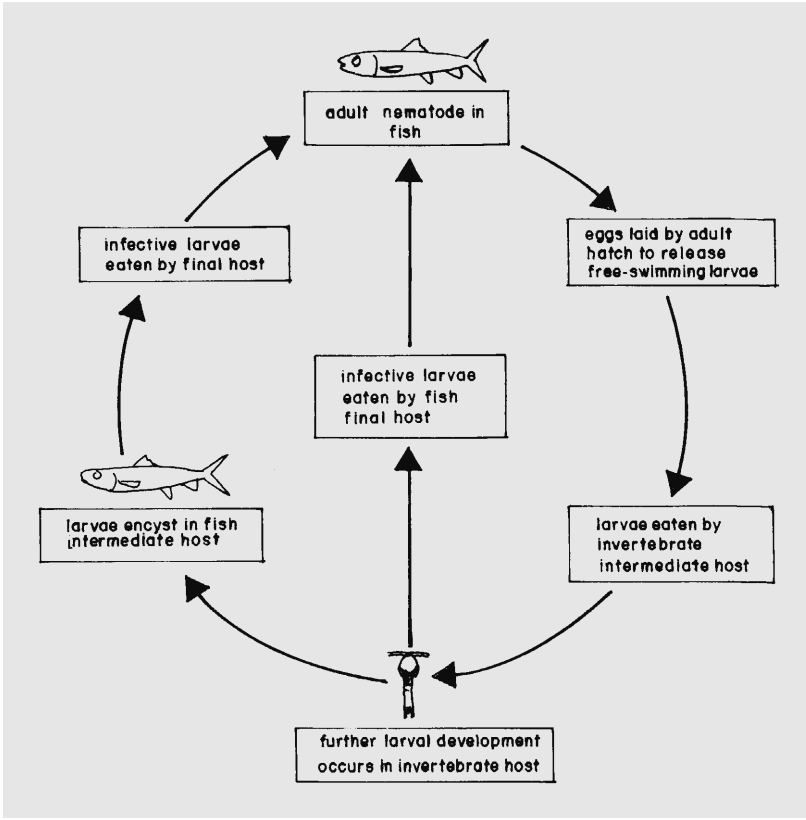

Figure 5-24. Life cycle of a parasitic nematode parasite

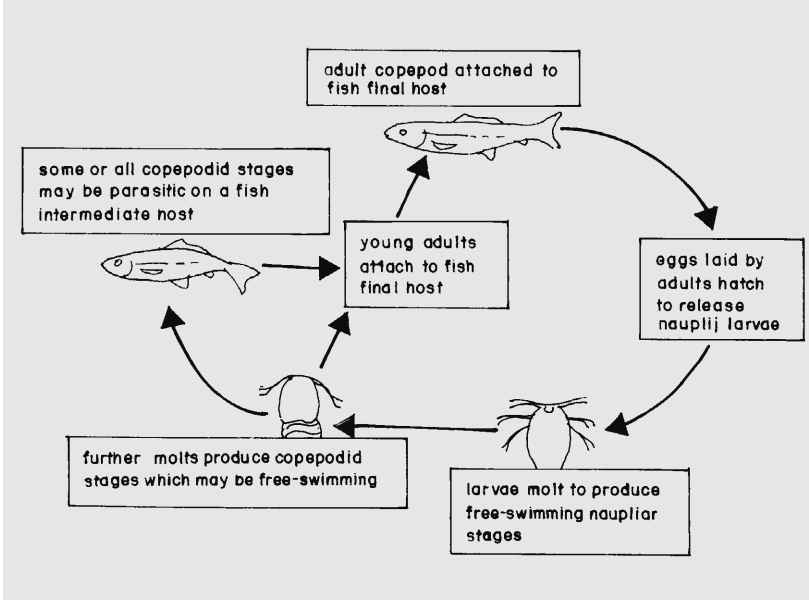

Figure 5-25. Life cycle of a parasitic copepod

ture female. "Gnathiid" isopoda are parasitic only during the larval stage known as praniza.

Leeches have a direct life cycle. Adults lay cocoons attached to a substrate. Young leeches hatch from cocoons and the life cycle is completed in more or less than a year's time.

SUMMARY

A wide variety of parasites have been identified as causing significant economic losses in fish and shrimp culture. Most of these parasites are difficult to control effectively with a single measure. The control of parasites is dependent on culture systems of the host fish, knowledge of the life cycle of the parasite, and the availability of effective treatment methods.

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6

CHAPTER SIX

Environmental and other non-infectious diseases

Gregoria Erazo-Pagador

Non-infectious diseases are caused by adverse environmental conditions, nutritional disorders, or genetic defects. While they can result in sudden mass mortality or death, they are not contagious.

Environmental diseases are the most important in aquaculture. These include low dissolved oxygen, high ammonia, high nitrite, or natural or man-made toxins in the aquatic environment.

This chapter presents some of the environmental and noninfectious diseases of aquatic animals.

FISH DISEASES ASSOCIATED WITH PHYSICO-CHEMICAL PROPERTIES OF WATER

Gas Bubble Disease

Gas bubble disease is due to supersaturation of dissolved gases (nitrogen or oxygen); supersaturation may be due to leaks in pump or valve systems in hatcheries; dense algal bloom that presumably caused oxygen depletion at night and supersaturation during the day.

SIGNS:

Affected fish show bubbles in the abdominal cavity, eyes, skin, gills, fins, mouth, swimbladder and within the digestive tract (Fig. 6-1) and exopthalmia. The clinical signs of gas bubble disease should not be confused with **Swimbladder stress syndrome** because the bubbles in the latter can be seen only in the swimbladder.

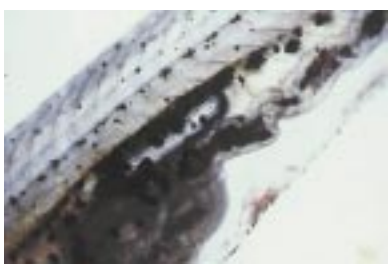

Figure 6-1. Gas bubble in the gut of milkfish fry

EFFECTS ON HOST:

Death due to embolism in blood and emphysema in tissues; edema and degeneration of the gill lamellae; bulging of the cornea; abrupt mass mortalities.

PREVENTION AND CONTROL

- Monitor dissolved oxygen (DO)
- Avoid algal blooms
- Maintain efficient operation of waterlines and pumps
- Sufficient water exchange

Swimbladder Stress Syndrome

Swimbladder stress syndrome (SBSS**)** is associated with malfunction of the swimbladder and is also associated with a combination of handling, high ambient temperature, high ambient illumination, dense algal bloom that presumably cause oxygen depletion at night and supersaturation during the day.

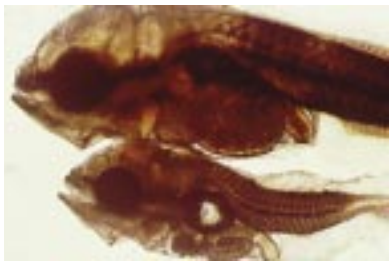

Figure 6-2. Seabass larva with Swimbladder stress syndrome compared with normal larva (top)

SIGNS:

Affected fish larvae show large bubble of gas in the region antero-dorsal to and outside the swimbladder (Fig. 6-2).

EFFECTS ON HOST:

Hyperinflation of swimbladder; high positive buoyancy and mass mortalities.

PREVENTION AND CONTROL:

- Filter rearing water
- Regulate algal bloom in hatcheries
- Provide strong aeration to maintain the larvae beneath the water surface in hatchery tanks

Asphyxiation/Hypoxia

Very low levels of dissolved oxygen (DO) cause asphyxiation/hypoxia.

SIGNS:

Affected fish gather at the water inlets and outlets. Also fish with wide gaping mouth swim at water surface and show rapid opercular movement.

PREVENTON AND CONTROL:

Monitor DO levels and provide aeration.

Salinity

Extremely high salinities are associated with progressive emaciation, scale loss, and opaque eye lenses in affected fish.

Alkalosis

Alkalosis comes about when water becomes too basic. The pH increases to a level higher than the fish can tolerate.

SIGNS:

Affected fish show corroded skin and gills and milky turbidity of the skin.

PREVENTION AND CONTROL:

Monitor pH level, and maintain the pH in a range optimal for the species being cultured.

Acidosis

Acidosis is caused by a drop in the pH to a level too low for the species.

SIGNS:

Affected fish show rapid swimming movements and gasping. Increased mucus secretion and death occur very quickly. Affected shrimp show poor growth, low molting frequency and yellow to orange to brown discoloration of the gill

and appendage surfaces. The pond soil turns reddish in color.

EFFECTS ON HOST:

Normal metabolism is hindered resulting in retarded growth and eventual death.

PREVENTION AND CONTROL:

- Monitor pH
- Apply lime and flush pond bottom before stocking

Sunburn Disease

Sunburn disease is due to excessive levels of ultraviolet irradiation from sunlight when fish are stocked in shallow uncovered raceways under intense sunlight.

SIGNS:

Development of gray focal circular ulcerative lesions on top of the head, pectoral, dorsal and upper tail fins.

EFFECTS ON HOST:

Ulcerative lesions may serve as portals of entry for other pathogens and may result in secondary infection.

PREVENTION AND CONTROL:

Place sunshades over ponds, and fish should be kept away from ultraviolet installations.

SHRIMP DISEASES ASSOCIATED WITH PHYSICO-CHEMICAL PROPERTIES OF WATER

Muscle Necrosis

Figure 6-3. *Penaeus monodon* juveniles with necrosis in the abdominal muscles

Figure 6-4. *Penaeus monodon* postlarva with "grainy" abdominal muscles

Muscle necrosis in shrimp is caused by temperature and salinity shock, low oxygen levels, overcrowding, rough handling and severe gill fouling.

SIGNS:

Affected shrimp show opaque white areas on the abdomen (Fig. 6-3); blackening on the edges of the uropod followed by erosion and liquid-filled boils at the tip of uropods in advanced stages; "wood grain" appearance of abdominal muscle in postlarvae (Fig. 6-4).

EFFECTS ON HOST:

There is a gradual death of cells of affected parts leading to erosion especially in the tail. This may then serve as portal of entry for secondary systemic bacterial infection.

PREVENTION AND CONTROL:

Reduce stocking density and improve water quality by daily water change (5-10%)

Bent/Cramped Tails or Body Cramp

Bent/cramped tails or body cramp in shrimps is associated with handling of shrimp in warm, humid air much warmer than culture water, and mineral imbalance.

SIGNS:

Figure 6-5. Tiger shrimp juvenile with body cramp

Affected shrimps have partial or complete rigid flexure of the tail (Fig. 6-5).

EFFECTS ON HOST:

Partially cramped shrimps swim with a humped abdomen; fully cramped shrimps lie on their sides at the pond/tank bottom. Healthy shrimps may cannibalize weak ones.

PREVENTION AND CONTROL:

Avoid possible causes.

Incomplete Molting

Incomplete molting is closely associated with low temperature of culture water.

SIGNS:

Figure 6-6. Incomplete molting in carapace of mysis stage larva (scanning electron microphotograph)

Presence of old exoskeleton attached to newly molted larvae, especially in appendages (Fig. 6-6).

EFFECTS ON HOST:

Abnormal swimming movement which could lead to easy predation and mortality.

PREVENTION AND CONTROL:

- Maintain optimum temperature in the rearing water
- Use water heater

Asphyxiation/Hypoxia

Reduced dissolved oxygen due to high organic load or algal bloom and subsequent die-off and high temperature

SIGNS:

Affected shrimp showed surface swimming and sudden mass mortality.

EFFECTS ON HOST:

Prolonged respiratory distress leads to death and sublethal levels may cause impairment of metabolism resulting in growth retardation.

PREVENTION AND CONTROL:

- Decrease stocking density
- Monitor water parameters frequently
- Provide aeration facilities and water pump for ready water change

Acidosis/Acid Sulfate Disease Sydrome

Figure 6-7. Pond with acidic soil

Acidosis is caused by low water and soil pH

SIGNS:

Affected shrimp show poor growth, low molting frequency and yellow to orange to brown discoloration of the gill and appendage surfaces. The pond soil turns reddish in color (Fig. 6-7).

EFFECTS ON HOST:

Normal metabolism is hindered resulting in retarded growth and eventual death.

PREVENTION AND CONTROL:

- Monitor the pH
- Apply lime and flush the ponds before stocking

Black Gill Disease

Figure 6-8. *Penaeus monodon* with black gill disease due to heavy siltation

Black gill disease is due to chemical contaminants, heavy siltation and ammonia or nitrite in rearing water; high organic load due to residual feed, debris, and fecal matter on pond bottom (i.e. black soil).

SIGNS:

The gills of affected shrimps show reddish, brownish to black discoloration and, in advanced cases, gill filaments become totally black (Fig. 6-8).

EFFECTS ON HOST:

Histological observations show that blackening of the gills may be due to the deposition of melanin at sites of tissue necrosis and heavy hemocyte activity.

PREVENTION AND CONTROL:

- Avoid heavy metal discharges of nearby factories from getting into the rearing facilities
- Remove black soil by scraping after each harvest and by draining pond water from the bottom during the culture period

Red Disease

Figure 6-9. *Penaeus monodon* with early signs of red disease

Red disease in shrimps is associated with high application of lime (2-6 tons/ha) in the pond that gives it a high initial pH; prolonged exposure to low salinity (6-15 ppt).

SIGNS:

Affected shrimps have red short streaks on gills or abdominal segments, yellowish to reddish discoloration of the body (Fig. 6-9) and increased fluid in the cephalothorax, emitting foul odor.

EFFECTS ON HOST:

Yellow to red discoloration in affected shrimps; histopathology of the hepatopancreas shows hemocytic infiltration in the spaces between the tubules; more advanced lesions are in the form of fibriotic and melanized encapsulation of necrotic tissues, either in the tubule itself or the sinuses around it.

PREVENTION AND CONTROL:

- Prepare pond bottom properly
- Reduce lime and organic matter content inputs

Chronic Soft-Shell Syndrome/ Soft-Shelling

Chronic soft-shell syndrome is associated with exposure of normal hard-shelled shrimps to pesticides and piscicides. Aquatin at 0.0154 - 1.54 ppm, Gusathion A at 1.5 - 150 ppb, rotenone at 10-50 ppm, and saponin at 100 ppm for 4 days can induce soft-shelling in initially hard-shelled stocks.

SIGNS:

Shell is thin and persistently soft for several weeks, shell surface is often dark rough and wrinkled, and affected shrimps are weak. The disease must not be confused with the condition of newly-molted shrimps, which have clean smooth, and soft shells that harden within 1-2 days.

EFFECTS ON HOST:

Affected shrimps are soft-shelled, grow slowly, and eventually die; histopathology of shrimps exposed to Gusathion A shows slight hyperplasia of the gill epithelium, delamination of the cells lining the tubules of the hepatopancreas, and general necrosis and degeneration of these tissues.

PREVENTION AND CONTROL:

- During pond preparation, flush ponds thoroughly particularly when pesticide contamination is suspected
- Maintain pond water and soil of good quality

DISEASES ASSOCIATED WITH PHYSICAL FACTORS

Diseases associated with physical factors are mainly due to handling, transport, high stocking density and predation. Secondary bacterial, viral, or parasitic infection may easily set in once injuries are introduced.

DIAGNOSIS OF ENVIRONMENTAL AND OTHER NON-INFECTIOUS DISEASES

Diagnosis of noninfectious diseases can be done through gross examination of fish for external/internal disease signs; histopathological/histochemical analysis; hematological analysis to assess the cellular composition of the blood in response to environmental stress; analysis of physico-chemical characteristics of rearing water; and evaluation of culture operations and management practices.

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7

CHAPTER SEVEN

Nutritional diseases

Celia R. Lavilla

Nutritional diseases of fish may develop as a result of deficiency (undernutrition), excess (overnutrition), or imbalance (malnutrition) of nutrients present in their food. The disease usually develops gradually because animals have body reserves that make up for nutritional deficiency up to a certain extent. Disease signs develop only when supply of any diet component falls below critical level. On the other hand, when there is too much food, the excess that is converted to fat and deposited in fish tissues and organs, may severely affect physiological functions of the fish.

TYPES OF FEEDS

Two types of diets are in use to nourish fish in aquaculture. Diets designed to add nutrients to food obtained from the pond or aquarium environment are called supplemental feeds. Feeds for intensively reared fishes that do not get nutrients supplied from the environment are called complete feeds. Complete feed formulas are based on the nutrient requirements of fishes, availability of the essential components, and digestibility of the ingredients used to prepare them. Various stages of fish require various sizes of feeds, thus, according to size, feeds are classified as: (1) larval feeds, (2) starter feeds, (3) grower feeds, (4) finisher feeds, (5) broodstock feeds, and (6) maintenance diets.

Supplemental or complete feeds may be prepared in moist, semi-moist or dry form. The type of ingredients used to prepare fish diets determines the type of diet. Moist rations are prepared from ingredients with high moisture content such as raw fish, meat products, wet vegetable products and similar ingredients. The moisture content of most moist rations is 70%. Semi-moist rations are prepared from dry products (dried fish products, cereal grains and other dry animal and vegetable products) that are added to ingredients with high moisture content. The final moisture content of semi-moist diets is approximately 35%. Dry rations are prepared from dry animal and vegetable products with a final moisture content of about 10%.

Larval fish must ingest and digest feed particles, and then absorb and utilize the released nutrients. Most research has focused on larval feed ingestion. Only now are researchers beginning to look at digestion, absorption, and utilization. It has been suggested that live food is needed because the prey contains digestive enzymes which help the larvae in digestion. They also contain relatively high levels of free amino acids that the larvae can easily absorb.

COMPONENTS OF FEEDS

Proteins and amino acids

Table 7-1 shows the types and biologic functions of proteins. The required concentration of protein in the feed will vary with species, size, age, water temperature, energy levels in the diet, and the quality of protein (digestibility and amino acid content) incorporated in the diet. Fish demand a balanced composition of amino acids in amounts that are adequate to sustain growth. Diets that are insufficient in essential amino acids may give rise to well-defined deficiency diseases. Table 7-2 lists the essential and non-essential amino acids. When used as the major protein source, processed fishmeal meets the dietary amino acid requirement. The use of mainly vegetable protein in a diet may make amino acid supplementation necessary. Protein is the most expensive ingredient in fish feed, and it may be tempting to economize on this ingredient. Too little protein will inhibit or retard growth, and may reduce resistance to disease.

Table 7-1. Types and biological functions of proteins

Type of Protein	Function
Enzymes	
DNA polymerase	repairs and replicates DNA
Lipase	digests lipids
Protease	digests proteins
Transport proteins	
Hemoglobin	transports oxygen in vertebrate blood
Hemocyanin	transports oxygen in invertebrate blood
Contractile proteins	
myosin and actin	contractile proteins of muscles
Protective blood proteins	
Antibodies	binds with foreign proteins
Fibrinogen and thrombin	blood clotting proteins
Hormones	
Insulin	regulates glucose metabolism
Structural proteins	
Keratin	components of skin, scales, etc.
Collagen	components of connective tissues

Table 7-2. Essential and non-essential amino acids for fish

Essential amino acids	
Arginine	Methionine
Histidine	Phenylalanine
Isoleucine	Threonine
Leucine	Tryptophan
Lysine	Valine
Non-essential amino acids	
Alanine	Glycine
Asparagine	Proline
Aspartic acid	Serine
Cysteine	Tyrosine
Glutamine	Glutamic acid

Carbohydrates

In crustaceans, carbohydrates either become stored as glycogen or form part of the structural make-up as chitin. Carbohydrates may be used as an energy supply by some species, but only up to a certain level. Most fish species have limited ability to metabolize carbohydrates, and have no nutritional demand for them. Since fish poorly utilizes carbohydrates, it eliminates the nutrient from the body in feces, and cause pollution concerns in fish farm sites. Some sources of carbohydrates for fish feeds are given in Table 7-3.

Table 7-3. Biological functions and sources of carbohydrates

Biological functions	
Storage polysaccharides :	Starch Glycogen
Structural polysaccharides :	Chitin Cellulose
Carbohydrate sources for fish feeds	
barley, grain	molasses, wood
bread flour	oats, grain
cassava meal	pea, seed
corn germ meal	rice bran
corn starch	rice grain
dextrin	rye, grain
linseed, flax	sorghum, gluten meal
molasses, beet	sorghum, grain
molasses, cane	wheat, grain
molasses, citrus	wheat, bran
molasses, corn	oats, hull

Fats and lipids

Lipids supply the fish with essential fatty acids that are necessary structural components of all cell membranes. Lipids are of great importance for physiological processes. They are also necessary for absorption of some vitamins, and constitute a relatively cheap energy source. The quality of lipids used in the diet should not be ignored. Polyunsaturated fatty acids are easily oxidized under storage especially under warm temperature. Rancid lipids are known to induce lipoid liver disease in fish.

Vitamins

Vitamins are complex organic substances that are essential to a wide variety of metabolic processes. They are only required in small amounts in the diet, but requirements for vitamins may increase during growth and spawning. Vitamins are divided into two classes: fat-soluble and water-soluble. Some vitamins like ascorbic acid are unstable in storage. This is why some feed manufacturers use more stabilized forms of ascorbic acid in their feed formulations. A listing of essential vitamins for fish as well as the forms of ascorbic acid used in fish feeds are given in Table 7-4.

Table 7-4. Essential vitamins for fish

Vitamins and their principal functions	
Vitamin A	Essential for normal vision
Vitamin D	Essential for bone formation
Vitamin E	Essential for membrane stability
Vitamin K	Essential for normal blood clotting
Thiamin	Co-factor in energy-yielding reactions
Riboflavin	Co-factor in metabolism
Pyridoxine	Co-factor in amino acid metabolism
Pantothenic acid	Co-factor in metabolism
Niacin	Co-factor in metabolism
Folic Acid	Essential for blood formation
Vitamin B 12	Essential for blood formation
Ascorbic Acid	Essential for collagen synthesis
Choline	Component of phospolipids, membranes
Myo-Inositol	Component of phospolipids, membranes
Forms of ascorbic acid used in fish feeds	
Crystalline	Very unstable
Fat-coated	Fat coating makes up 30% of weight Losses occur during extrusion; unstable during feed stage
Ascorbate-2-sulfate	Very stable; low availability for many species
Ascorbate-2-phosphate	Very stable; high availability

Minerals

Fish require minerals for maintenance of osmotic balance between body fluids and their environment. Seven major minerals are required in large amounts and constitute 60-80% of all inorganic materials in the body. The seven minerals are calcium, phosphorous, sulfur, sodium, chlorine, potassium, and magnesium. Trace minerals are just as essential as major minerals, but are needed only in small amounts. The nine essential trace minerals are iron, copper, manganese, cobalt, zinc, iodine, molybdenum, fluorine, and selenium.

Pigments

Pigments added to feeds result in an attractive red flesh color in salmonids. Quite large levels of shrimp meal in the diet can achieve good results as well. Feed manufacturers usually include synthetic analogues of the naturally occurring pigment asthaxanthin or cathaxanthin as additives.

Antioxidants, binder, and other feed components

Fish feeds contain high levels of unsaturated oils that easily oxidize resulting in breakdown of oils and other nutrients. This can be controlled by adding antioxidants such as butylhydroxytoluene (BHT), butylhydroxyanisole (BHA), ethoxyquin, and tocopherol (vitamin E). The levels of BHT, BHA, and ethoxyquin allowed in feeds by regulations often are not adequate to control oxidation of the high levels of unsaturated oils in fish feeds. When additional antioxidants are needed, feed formulators should supplement with vitamin E to the levels permitted to protect the oils in fish feeds.

Fiber is an indigestible dietary material derived from cell walls. In concentrations of less than 8% fiber may add structural integrity to pellet feeds. Larger amounts often impair pellet quality. Carbohydrates are important binders in commercial fish feed production. Taste or palatability of the ration for each of the fish species must also be considered. Complete feed rations include attractants to draw fish attention to the feed.

DETERMINING THE NUTRITIONAL STATUS OF FISH

Nutritional deficiency signs, like those listed in Table 7-5, are useful in diagnosing feed problems. However, the absence of visible signs does not necessarily mean that the fish is in optimal or desirable nutritional health. Increasing the dietary level of all essential nutrients result in a heightened physiological response up to the point at which the dietary requirements are met. Beyond a certain level of nutrient availability, concomitant increase in physiological response is not seen. This is the dietary level that supports optimal nutritional status. At some level below that requirement, visible deficiency signs appear in the fish. The range of dietary intake between that resulting in visible signs of deficiency and that resulting in no attendant increase in physiological function is an area referred to as subclinical deficiency. Fish receiving this level of an essential nutrient do not show signs of deficiency, yet they may be nutritionally compromised and less able to resist infectious disease or cope with suboptimal water quality conditions. These points are illustrated by Hardy (1991) in Figure 7-1.

Diagnosis of nutritional diseases requires knowledge of the following:

a. External and internal disease signs:
b. Quantitative clinical chemistry of blood or tissue of diseased fish;
c. Histopathological examination;
d. Histochemical analysis; and
e. Proximate analysis of the diet given.

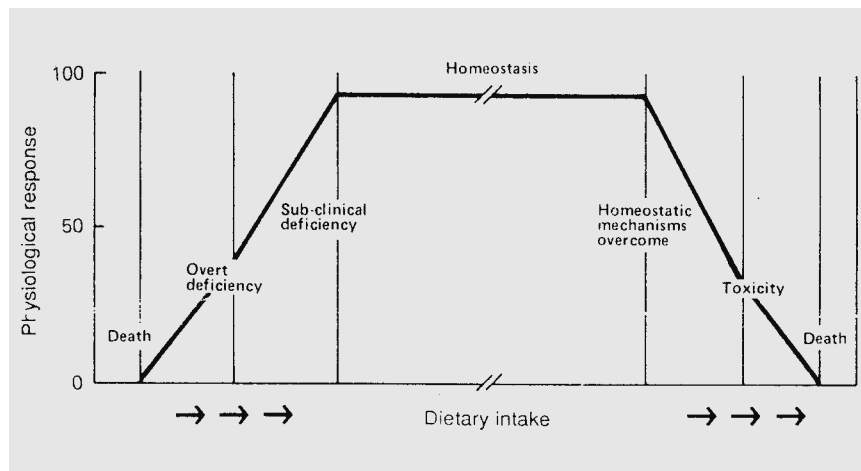

Figure 7-1. The relationship between the dietary intake of an essential nutrient and the physiological response of an animal or fish

Table 7-5. Summary of nutritional deficiency signs commonly observed in fish

Signs	Possible nutrient deficiencies
Anemia	Folic acid, inositol, niacin, pyridoxine Riboflavin, vitamins A, B_{12}, C, E, and K
Anorexia	Biotin, folic acid, inositol, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine, vitamins A, B_{12} and C.
Ascites	Vitamins A, C, and E
Atrophy, gills	Pantothenic acid, vitamin A
Atrophy, lateral muscle	Biotin, thiamine, vitamin E
Cartilage abnormality	Vitamins C and A, tryptophan
Cataract	Methionine, riboflavin, thiamine, zinc
Cloudy lens	Methionine, riboflavin, zinc
Clubbed gills	Pantothenic acid
Decoloration, skin	Fatty acids, thiamine, vitamin A
Deformation, bone	Phosphorus, vitamin A
Deformation, lens	Vitamin A
Disease resistance, low	Protein, vitamin C
Dystrophy, muscular	Selenium, vitamin E
Edema	Niacin, pyridoxine, thiamin, vitamins A and E
Equilibrium loss	Pyridoxine, thiamine
Erosion, fin	Fatty acids, riboflavin, vitamin A, zinc
Exophthalmia	Pyridoxine, vitamin A, C, and E
Fatty liver	Biotin, choline, fatty acids, inositol, vitamin E
Feed conversion, poor	Vitamin E, biotin, calcium, choline, fat, folic acid, inositol, niacin, protein, riboflavin
Fragility, fin	Folic acid
Growth, poor	Biotin, calcium, choline, fat, folic acid, inositol, niacin, pantothenic acid, protein, pyridoxine, riboflavin, thiamin, vitamins A, B_{12}, C, D, and E
Hemoglobin, low	Iron, vitamins A, B_{12} and C
Hemorrhage, eye	Riboflavin, vitamin A
Hemorrhage, gill	Vitamin C
Hemorrhage, kidney	Choline, vitamins A and C
Hemorrhage, liver	Vitamin C
Hemorrhage, skin	Niacin, pantothenic acid, riboflavin, vitamins A and C
Lethargy	Folic acid, niacin, pantothenic acid, thiamine, vitamin C
Lipoid liver	Fatty acids, rancid fat
Lordosis	Vitamin C
Low glycogen, liver	Vitamin C
Myopathy, cardiac	Essential fatty acids
Necrosis, liver	Pantothenic acid
Pinhead	Starvation
Pigmentation, iris	Riboflavin
Scoliosis	Phosphorus, tryptophan, vitamins C and D
Swimming, erratic	Pyridoxine, pantothenic acid
Vacuolation, liver	Vitamin C

Information on the feeding practices, feeding rates, storage conditions of feeds, and feeding behavior of the fish are also important.

Diagnosis of dietary carbohydrate-related pathology can be made by blood glucose and liver glycogen analyses. Histopathological examination of stained tissue sections can be useful in the diagnosis of nutritional diseases. Diagnosis of vitamin deficiencies is difficult. Gross signs, blood chemistry, hematology, tissue vitamin assay, gross pathology, histopathology and ration vitamin assay may be necessary for making a diagnosis.

NUTRITIONAL DEFICIENCY DISEASES OF FISH

Deficiency diseases in fish occur when the tissue reserves have been depleted and the dietary supply of any necessary nutrient falls below the level that supports optimum nutritional status. Table 7-5 shows the common deficiency signs observed in fish. It is important to realize that by the time signs of nutritional deficiency are visible, fish have usually stopped eating, and feeding fortified diets will not completely reverse the condition in the fish population and prevent losses. Several pathological syndromes have been reported in fish.

Amino acid deficiency syndrome

Like other deficiency diseases, diagnosis of malnutrition involving protein or amino acid deficiencies is extremely difficult because many indications of deficiency are non-specific. Many of the same diagnostic findings noted in protein and amino acid deficiency are similar to physical and functional alterations found in the diagnosis of diseases caused by other etiological agents. Probably the most common sign of protein and/or amino acid deficiency in fishes is reduction or cessation of growth. Limiting even one indispensable amino acid will affect growth. Feeding a diet in which an essential amino acid has been removed will cause growth to cease until the amino acid is restored to the diet.

Fats and lipids deficiency

Among the clinical features manifested by fish with essential fatty acid deficiency are depigmentation, fin erosion, cardiac myopathy and fatty infiltration of the liver. Lipoid liver disease is usually seen in fish fed on trash fish or pelleted diets in which part of the lipid component has gone rancid. Rancid lipids react with protein to lower its biological value and have a deleterious effect on those vitamins which are not themselves antioxidants. Fish suffering from lipoid liver disease have extreme anemia (manifested by pallor of the gills), bronzed rounded heart and a swollen liver with rounded edges.

Vitamin imbalances

Depletion of the body storage of any single vitamin can be responsible for specific or general disease signs. Disease signs and gross pathology usually suggest which vitamin may be deficient, and other diagnostic procedures are then used to complete the diagnosis. Results of research in which specific vitamins have been reduced or eliminated from the diets of fishes have demonstrated the

usual signs, gross pathology and histopathology to be expected from specific vitamin deficiency (Table 7-5). Nutritional myopathy syndrome in cultured fishes in Japan is a disease characterized by necrosis and degeneration of the lateral musculature of fish. It has been associated with vitamin E deficiency and with lipid peroxides in the diet. Supplementation of vitamin E was confirmed to prevent myopathic changes in carp.

Mineral deficiencies

Mineral deficiency studies with fishes are difficult because all traces of each mineral to be studied must be removed from both food and water. Thus, only a limited number of mineral deficiencies are known or have been studied in fishes. The role of calcium, potassium, and magnesium in bone and joint metabolism is well recognized and there is evidence that imbalances or deficiencies of these can result in spinal abnormalities which leave the fish deformed thereafter.

NUTRITIONAL DISEASES OF SHRIMPS

Feeding farmed shrimps generally depends on the culture system employed. The primary goal is to provide balanced nutrients to the farmed population and this is influenced by the quality and quantity of the nutritional resource and biomass of shrimps that rely on this as a source of food. Thus, in extensive production systems, feed is often not applied or the quality, in terms of nutrient composition, is low because the farm population can derive sufficient levels of balanced nutrition from the pond system. However, at higher stocking and biomass, the ability of the pond environment to sustain shrimp growth becomes limiting. At this point balanced nutrition of the applied feed is essential for continued growth of the population. Hence, the importance of nutritional quality of feeds is a function of the type of culture system and the shrimp biomass within the system. For obvious reasons, nutritional deficiency disease is much more important in semi-intensive through super intensive systems than it is in extensive culture. The following nutritionally-related diseases or syndromes have been observed in cultured shrimps:

Vitamin C deficiency

Vitamin C deficiency or black death disease occurred in juvenile to subadult penaeid shrimp cultured intensively in tanks. All penaeid species are considered susceptible. The cause is low level of vitamin C in the diet, and lack or absence of algal growth or other vitamin C sources in the culture system. Presence of black lesions below a non-ulcerated cuticle provides a presumptive diagnosis of the disease. Diagnostic confirmation is done by histological demonstration of the characteristic melanized hemocytic nodules within tissues of high collagen content such as the connective tissues beneath the shell. Bacterial septicemia often accompanies the disease, suggesting an important role of vitamin C in crustacean defense to infection. Experimental results showed that vitamin C-deficient shrimps had slower wound-healing process than shrimps with adequate vitamin C in their diet.

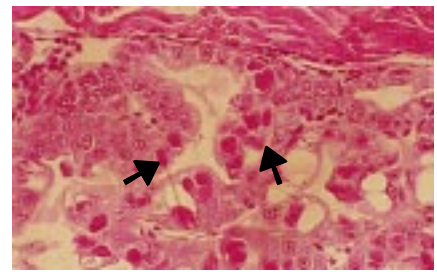

Figure 7-2a. Histological section of the hepatopancreas of shrimp juvenile at the start of the experiment showing numerous monodon baculovirus (MBV) occlusion bodies (arrows) (Hematoxylin and Eosin, 200x)

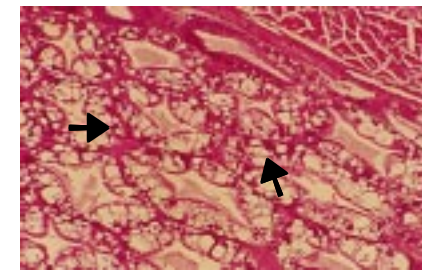

Figure 7-2b. Histological section of the hepatopancreas of shrimp juvenile fed a diet with 100 ppm phosphated ascorbic acid. Note the absence of MBV occlusion bodies and the presence of numerous storage vacuoles (arrows) (Hematoxylin and Eosin, 200x)

Shrimps in vitamin C-deficient populations had reduced growth and are prone to shell disease. Under experimental conditions, monodon baculovirus (MBV)-infected shrimps fed diets with 100 ppm phosphated ascorbic acid (MAP) or higher showed a marked absence of infection after three months (Fig. 7-2a, b) Shrimps fed vitamin C-deficient diets in the same experiment had MBV occlusion bodies still evident in the hepatopancreatic tubules after the same test period. The extent of vitamin C deficiency in cultured crustacean populations is unknown, but subclinical vitamin C deficiency may be widespread and common in intensive culture settings. Once identified as a problem, control is achieved through proper diet formulation to deliver sufficient vitamin C needed for shrimp growth. Highly stable forms of vitamin C are presently available for use, thus the problem of vitamin C degradation during feed manufacture and storage should no longer be a problem.

Chronic soft-shell syndrome

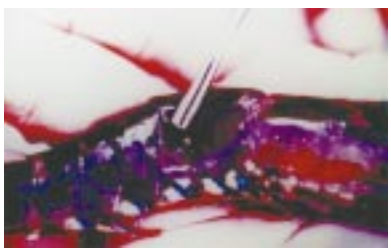

Figure 7-3. Pond-reared *Penaeus monodon* juvenile with chronic soft-shell syndrome

Soft-shell syndrome (Fig. 7-3) is a condition noted in farmed *Penaeus monodon* in the Philippines, India and probably other areas within the culture range of this species. In regions where it occurs, the syndrome is recognized as a significant problem that adversely affects production of grow-out farms. Soft-shelled shrimps are weak, prone to epibiotic fouling and cannibalism, and have reduced market value. The soft-shell condition results with the delay of several weeks in the normal hardening process of the cuticle following molting. Although the presence of the soft-shelled condition correlated well with the environmental conditions of high soil pH, low water phosphate, low organic matter, and insufficient water exchange, inadequate feeding practices or insufficient food supply are the primary causes of the soft-shell problem. The atrophied condition of the hepatopancreas of soft-shelled shrimps support the hypothesis of underfeeding as a cause for the syndrome. Infrequent water exchange would support this conclusion because lack of an adequate supply of water leads farmers to reduce feed inputs because of instability in water quality conditions and greater likelihood of losses due to oxygen problem.

Blue Disease or blue shell syndrome

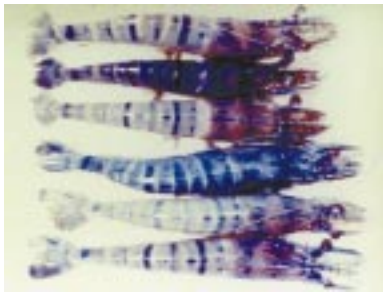

Figure 7-4. *Penaeus monodon* adults with blue shell syndrome (2nd and 4th from left

Shrimps with this disease/syndrome are light blue rather than grayish-brown in color (Fig. 7-4). Although the syndrome is not well understood until now, a deficiency in dietary carotenoids (i.e. asthaxanthin) is believed to be one of the causes. Prevention and control of blue disease is achieved through dietary manipulation. Future work is needed to provide practical guidelines to farmers on management of the syndrome. Basic research work to further understand the effect of carotenoid dietary deficiency on crustacean health is also important. The role of environmental factors in the development of the syndrome also needs to be investigated.

Body cramp or cramped tail syndrome

Body cramp refers to a syndrome of uncertain etiology. Nutritional factors, such as an imbalance in the Ca:Mg ratio and vitamin B deficiency, have been implicated as etiologic agents. Body cramp and muscle necrosis often occur together with the striated muscles as the target organ. Body cramp describes a functional lesion in which the muscle contracts but is unable to relax, hence, the abdomen or tail remains flexed for an extended period. The role of environmental factors in the development of this syndrome are presented in Chapter 6.

Underfeeding

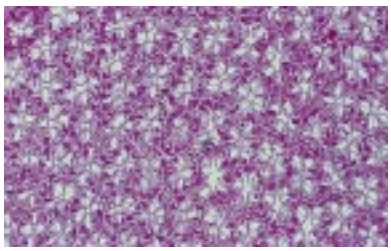

Figure 7-5a. Normal structure of the hepatopancreas of juvenile shrimp showing well-vacuolated cells lining the tubules (Hematoxylin and Eosin, 40x)

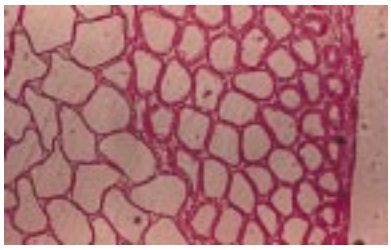

Figure 7-5b. Hepatopancreas of underfed shrimp juvenile showing severely atrophied tubules due to the absence of stored food (Hematoxylin and Eosin, 40x)

Underfed shrimps appear to have loose and oversized shells due to the reduction of the muscular mass in the abdominal region. The most prominent lesion associated with underfeeding appears in the hepatopancreas. There is a marked atrophy of the tubules due to the absence of stored food in the cells (Fig. 7-5a, b). Underfeeding usually results because of management inexperience. This happens when regular monitoring and estimation of shrimp biomass is not carried out, or the feeding rate is inappropriate because the animals are overstocked. Underfeeding could also be due to inadequate availability of energy to sustain growth of the shrimps in the pond, or a lack of specific, limiting nutrients necessary for growth. In either case the initial sign is reduction or lack of growth of the population in the production setting. A diagnosis of underfeeding requires careful review of production records, water quality monitoring data, and animal examination. Since the principal sign of reduced or no growth is non-specific, many factors must be considered and eliminated in the diagnostic process.

Prevention and control of an underfeeding problem is based on identification of the underlying contributing conditions and correction of these deficiencies. For example, if feeding rates are reduced to prevent water quality problems, then increased water exchange, reduction of anticipated harvest biomass, or both are needed. Improved growth of shrimps following a change in feed or feeding regimen supports the diagnosis of an underfeeding problem.

FEED QUALITY PROBLEMS WITH HEALTH IMPLICATIONS

Improper storage

Feed storage is of particular importance in the tropics where the warm humid weather promotes rapid deterioration of feeds. Feeds in bags should be stored in a cool, dry area and low humidity must be maintained because moisture enhances mold growth and attracts insects. If proper storage conditions are not maintained, several factors can cause spoilage of the feed and pose problems such as:

- loss of vitamins and nutrients
- rancidity
- contamination with microorganisms or their metabolic products

Artificial feeds contain ingredients that are prone to become rancid like fish meal, copra meal, rice bran and fish oils. Rancid oils can be toxic, may destroy other nutrients, and will cause off-flavor of the feed. Rancidity in feeds has a negative effect on its palatability, nutritional value, and it may also lead to toxicity problems. Fish that have prolonged exposure to rancid feeds may exhibit reduced appetite, slow growth and low feed efficiency.

Another feed-related problem with fish health implication is connected with the use of ingredients that are contaminated with microorganisms which may produce various toxins. This is primarily a problem with fish meal and other animal by-product meals, which are contaminated with *Salmonella*. Avoiding this problem involves the establishment of standards for fish feed ingredient quality. The feed standards could include maximum acceptable levels of microbial contamination.

Aflatoxin contamination

Molds like those belonging to genus *Aspergillus*, which grow on the feeds, cause spoilage. These may also produce toxins such as aflatoxin. While aflatoxin may be produced during feed storage, it may also be produced in feed ingredients before these are used to make feeds. So, the establishment of standards of feed ingredients must include examination of suspected materials for aflatoxin. In feeds that are susceptible to mold formation during storage, the addition of mold inhibitors is a recommended practice. The chosen inhibitor should not affect the palatability of feed to fish.

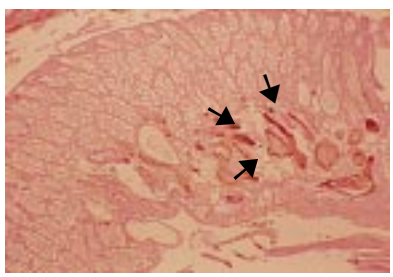

Figure 7-6. Hepatopancreas of shrimp fed diets with 200 ppb aflatoxin B_1 showing melanized tubules in the central region (arrows) (Hematoxylin and Eosin, 40x)

Aflatoxicosis in shrimp

Because of the rampant occurrence of red disease-like lesions in cultured shrimp in the early 1990s, a study on the possible role of aflatoxin B_1 ($AFLB_1$) on the development of the disease was conducted. Juvenile shrimps were given diets with various levels of $AFLB_1$, namely: 25, 50, 75, 100, and 200 parts per billion (ppb) for two months. Control shrimps were fed complete diets without $AFLB_1$. The results showed that shrimps fed diets containing $AFLB_1$ of 75 ppb and above had comparatively poor growth rates. In fact, shrimps given diets with 200 ppb $AFLB_1$ showed negative growth after two months. The primary organ that is affected in aflatoxicosis is the hepatopancreas. Melanized lesions develop primarily in the central region of the organ causing dysfunction (Fig.

7-6). As the hepatopancreas is the organ responsible in the digestion, absorption and storage of food, damage in its tissues leads to slow growth. Shrimps exposed to $AFLB_1$ were also prone to shell disease.

Studies also show that *P. monodon* juveniles can tolerate aflatoxin B_1 levels of up to 52.3 µg/kg feeds, but histopathological changes are usually evident in shrimp tissues at a level of 26.5 µg/kg. Growth retardation was observed at levels of more than or equal to 73.8 µg/kg feeds.

Toxic components of the diet

Naturally occurring organic compounds found in some feed ingredients produce toxic responses in fish. Tannic acid and cyclopropenoid fatty acids induce liver cancer in fish. Gossypol, a toxin present in untreated cottonseed meal, causes anorexia and ceroid accumulation in the liver. Phytic acid, which ties up with zinc in the feed, and growth inhibitors found in soybean meal can be destroyed by proper heating during processing. Chlorinated hydrocarbons occur as contaminants in fish meal and can cause mortality when present in fry feeds. Broodstock transfer these compounds from the feed to their eggs, resulting in low hatchability and high mortality of fry. The environment and feeds should be free from toxicants to maintain the health and efficient production of fish. Feed ingredients may also contain anti-nutritional factors. Most of these factors are found in ingredients of plant origin. The most common anti-nutritional factors in feed ingredients are trypsin inhibitors found in soybean meal.

The use of leaf meals as non-conventional protein sources for shrimp diets has been studied. *Leucaena leucocephala* (called "ipil-ipil" in the Philippines) leaves are one of the promising sources of plant protein for shrimp diets, however, the leaves contain a poisonous amino acid, mimosine, which causes pathological changes in the storage cells of the shrimp's digestive organ. The mimosine content of the *Leucaena* leaves could be reduced by 70% if the leaves are soaked in freshwater for 24 h prior to incorporation in the artificial feeds.

Transmission of diseases through trash fish

The use of fresh or raw trash fish as a food presents the possibility of transmitting specific fish pathogens directly. Viral and bacterial diseases, as well as parasites can be transmitted to healthy fish by feeding them with contaminated trash fish. This problem will be minimized if trash fish is stored frozen in single-ration packages. Microorganisms and parasites that do not survive freezing temperatures will be eliminated. Thawing and re-freezing trash fish should be avoided to prevent their spoilage. Trash fish that have gone through a heating step to destroy any fish pathogen that might be present is more appropriate than raw fish.

SUMMARY

Diagnosis of nutritional diseases is difficult because many signs exhibited by fish are non-specific and most nutritional deficiencies are hard to define. A compilation of data on feed composition and feeding management, as well as husbandry practices, are needed to define a case. Most of data on fish and

shrimp nutritional diseases were gathered under experimental conditions. Under farm conditions, most of that definition would be clouded with errors in husbandry practices or secondary infection. Therefore, attempts to diagnose nutritional diseases should be carefully done using every available technique to define the case.

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8

CHAPTER EIGHT

Physical, environmental, and chemical methods of disease prevention and control

Erlinda R. Cruz-Lacierda and Gregoria E. Erazo-Pagador

The environment determines the balance between the host and the disease agent. Microorganisms are always present in the water and some of them cause disease only when the host have been weakened through exposure to stressful environmental conditions. Thus, the fish culturist must be able to maximize the environment and make it favorable to the cultured species.

This chapter deals with the general principles involved in the physical, environmental and chemical aspects of disease prevention and control that are applicable in the hatchery and the grow-out stages of shrimps and fishes. Specific chemical treatments against a particular disease are discussed in their respective chapters.

PHYSICAL METHODS

Physical methods of disease prevention and control are based on the physiological tolerance of disease agents to adverse conditions such as increased or low temperature, absence of moisture, presence of deleterious irradiation; and the removal of pathogen sources or presence of physical barriers to prevent contact between the disease agent and the host.

Potential pathogens can be removed by ultraviolet radiation and through the process of microfiltration. Chemical pollutants can be eliminated by carbon filtration, biofiltration and water dilution. Exposure of tank or pond to heated water and sun-drying can also eliminate some microbial flora. Infected fish must be removed quickly and destroyed.

Such health classification scheme has been proposed by Ghittino and de Kinkelin (1975):

- Fish free of specific pathogenic organisms (SPF) – refer to fish free of all species-specific pathogens. The water supply must be completely sterile and exchanges of fish is possible only between SPF classified establishments.

- Fish free of coded pathogenic organisms (CPF) – include fish free of all diseases appearing in a list drawn up by an international agreement. For Southeast Asian countries, such a list has yet to be drafted. Water supply would have to be pretreated. CPF classified farms can receive SPF or CPF fish but cannot dispatch fish to SPF farms.
- Fish free of specified diseases (SDF) – relate to fish reared in water supplies in which pathogens could exist, multiply or be disseminated by wild fish. Disease could occur but readily controlled by therapy. Certification for freedom from certain diseases can be issued but guarantees only for the diseases listed in the document. Such a farm can receive fish from SPF or SDF farms as well as enterprises of similar sanitary level.
- Uncontrolled fish consist of fish not checked for the presence of disease or pathogens. Fish exchange is possible only with farms of similar category but can receive fish from the three foregoing ones.

This sanitary classification of fish farms can be used as basis for issuance of permits for fish import, export, exchange or restocking.

The International Council for the Exploration of the Seas (ICES) have recommended policy measures dealing with the introduction of aquatic species and guidelines for implementation, including methods to minimize the possibility of disease transfers. Such recommendation is the Revised Code of Practice to Reduce the Risks of Adverse Effects Arising from the Introduction and Transfers of Marine Species (Sinderman and Lightner, 1988). The ICES Code of Practice is as follows:

- A recommended procedure for all species prior to reaching a decision regarding new introductions;
- Recommended action if the decision is taken to proceed with the introduction;
- A suggestion that regulatory agencies use the strongest possible measures to prevent unauthorized introductions;
- A recommended procedure for introduced or transferred species which are part of current commercial practice; and
- A note recognizing that countries will have different attitudes toward the selection of the place of inspection and control of the consignment.

ENVIRONMENTAL METHODS

Monitoring of the environment is extremely important for success in fish culture. The primary objective of the environmental method is to protect the host by intercepting the pathogen or cutting its pathway to the host. The sections that follow will attempt to describe some of the ways this is carried out.

Proper Hatchery/Pond Design

Trained personnel and well-designed hatcheries or ponds are important requirements in ensuring that fish health management practices can be incorporated in the routine operations of an aquaculture system. The hatchery or farm should have access to a good water supply free from any type of pollution (Fig. 8-1). Pond development, wherever possible, should be adjacent to mangrove areas for protection from erosion, and to provide natural filter for farm effluent. Provision of independent water supply and drainage canals (Fig. 8-2) to each individual part of an aquaculture grow-out facility will ensure that water emerging from one pond compartment does not enter the other compartments. Fishponds should be kept free of wild fish and other potential carriers of infectious agents such as invertebrates, pests and predators. The farm/hatchery should be accessible by road to avoid excessively long transport time of the larvae or fish.

Good Water Quality

Good water quality is crucial in the hatchery or pond; it can spell the difference between success and failure of the aquaculture enterprise. The lower the water quality, the fewer fish/shrimp it will support; the higher the water quality, the higher the production potential will be. Aside from being pathogen-free, the

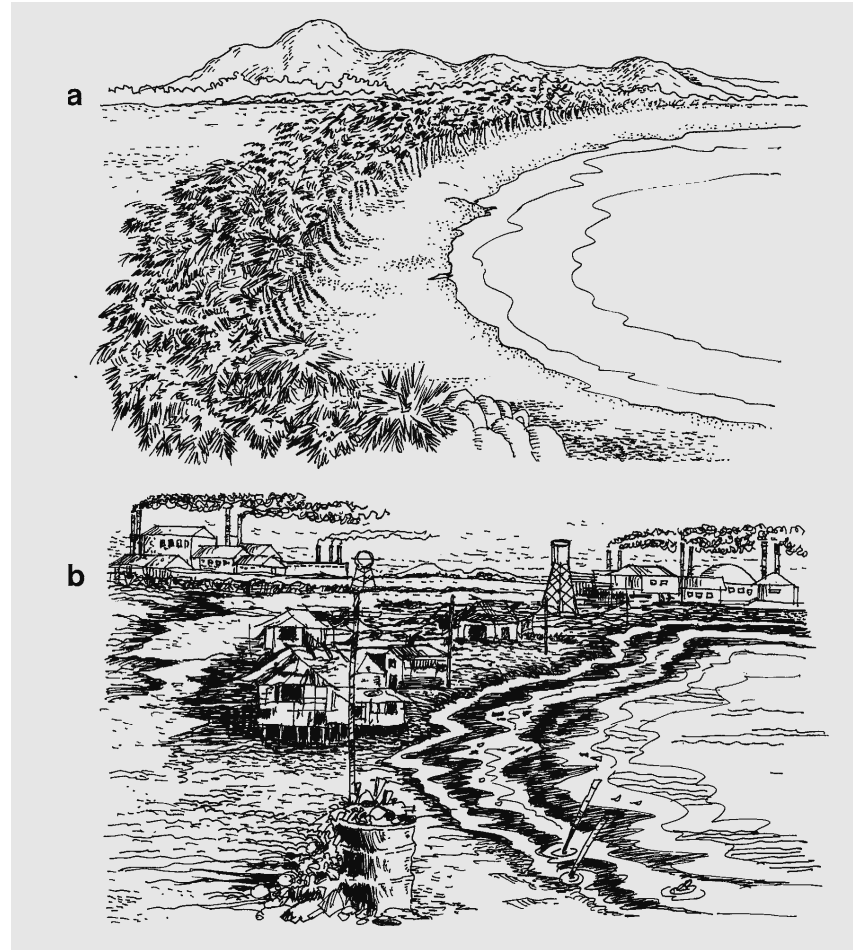

Figure 8-1. Ideal (a) and poor (b) hatchery sites; the latter is polluted with industrial and domestic wastes

water must meet the specific quality requirements of the cultured species. Monitor regularly the rearing water quality parameters such as salinity, pH, dissolved oxygen, ammonia and temperature. Ultraviolet radiation and filtration systems eliminate potential pathogens. Sand filters or filter bags will remove most of the debris.

Filter water with fine net (Fig. 8-3), cloth or cartridge filter before stocking in tanks. Clean filters regularly. Aerate and change rearing water regularly. Siphon off bottom sediments regularly to remove feces, organic debris and unused feed. Provide paddle wheels as aeration in ponds and a large settling reservoir to reduce the organic and particulate load before it is directed into ponds.

Sanitary Practices

Cleanliness improves the general standard of health. It also prevents or retards the development of disease agents. Drain and dry the tank (Fig. 8-4) and pond bottom in between culture periods. Backwash or clean filters regularly (Fig. 8-5). Day-to-day hygiene measures should include siphoning out of organic material that accumulate in tank bottom, immediate removal of any dead fish, and careful control of aquatic vegetation in ponds. Provide properly labeled gear like scoop nets, buckets and pails for exclusive use in individual facilities. Use PVC or non-toxic plastic pipes, pails and other equipment parts. Workers should disinfect their hands with soap and water before preparing and administering feed, and before performing other jobs.

Stress Avoidance

Stress plays a major role in the susceptibility of fish to disease. Most diseases are stress-related. Poor water quality, inadequate food, overstocking, handling, grading, and transfer and transport of animals are stress inducing factors. Regular monitoring of the health status of the stock can detect early signs or onset of diseases before they become uncontrollable.

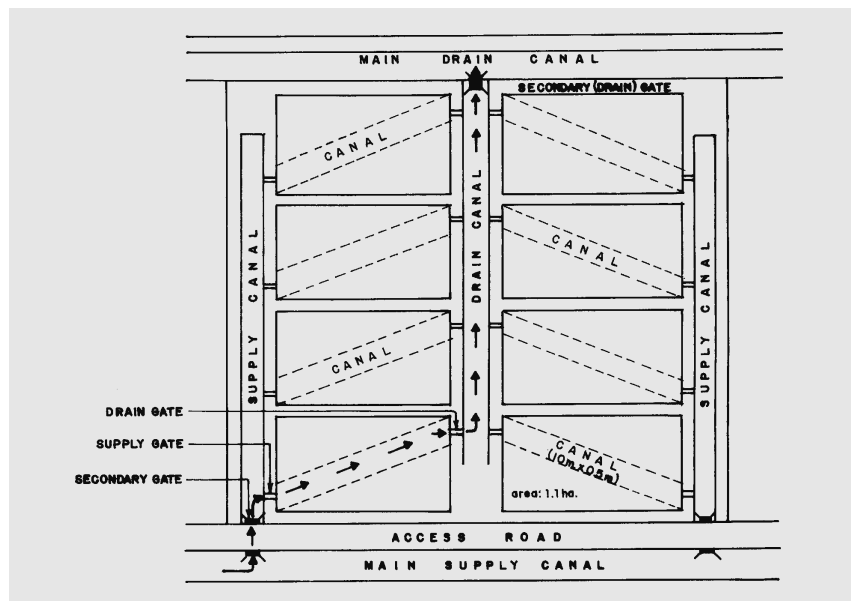

Figure 8-2. Lay-out of a 10-ha semi-intensive shrimp pond system

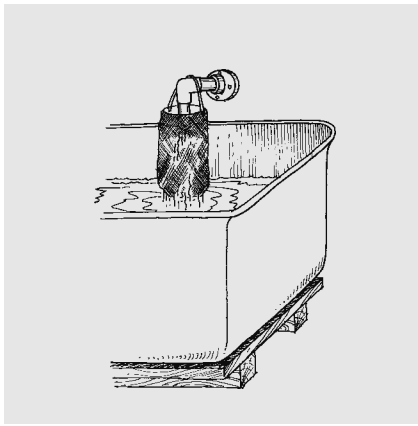

Figure 8-3. Filtration of water using fine net

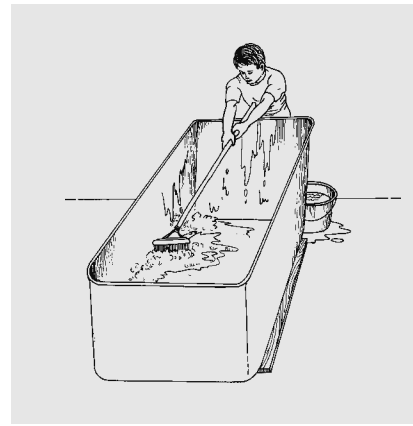

Figure 8-4. Thorough cleaning of rearing tank with stiff brush

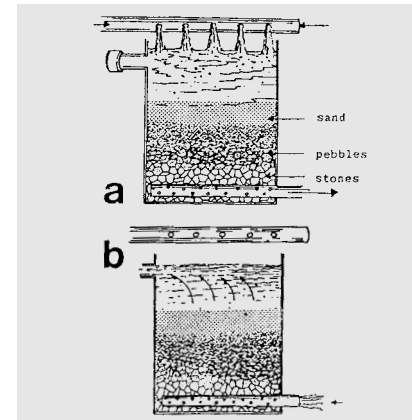

Figure 8-5. Sand filter system showing operationa inlet flow (a) and reverse flow or backwashing (b)

Quarantine Procedures/ Legislation

Quarantine measures are very important for the prevention of the international spread of diseases of aquatic organisms. Legislations to impose quarantine procedures on fish imported and exported requiring health certification of incoming fish into countries will minimize worldwide spread of fish pathogens. Sanitary classification of farms can be instituted such that exchanges of fish occur only among farms of similar fish health status.

Quarantine should be practiced to minimize risk of disease among local species. Fish imported from abroad, or fish moved from one place to another within a country, should be placed in quarantine on arrival and should remain there until all danger has passed. The quarantine period should exceed the length of the longest latent period of the pathogens. Fish markets can become centers for the dispersal of pathogens. To avoid this danger, fish should be disinfected upon arrival at the market. The quarantine period for incoming stock must be observed for at least 2-3 weeks. Legislation of quarantine requirements should be imposed on all imported and exported fish to minimize the spread of disease, both within a country and outside. Quarantine ponds must be safely isolated and must be located downstream from all other ponds on the farms to minimize the danger of pathogen penetration.

Termination Procedures

Termination procedures may also be used to control fish diseases. These include destruction of infected individuals, by burning, cooking or burying in limed pits. Disposal of infected individuals should be to areas that will not affect the culture system. Avoid contact between diseased and normal individuals. Disinfect the water supply system that may have carried the pathogen by draining and drying the affected tanks and ponds. Disinfect paraphernalia used on infected individuals.

CHEMICAL METHODS

Prophylactic Methods

Prophylactic treatment methods are protective or defensive measures designed to prevent a disease from occurring. They are used to combat external parasites and stress-mediated bacterial diseases.

Disinfecting culture facilities

Tanks – Rearing tanks should be disinfected in between rearing periods. Drain and scrub tank bottom and sidewalls using powdered detergent and plastic brush to remove debris. Rinse thoroughly to remove soap suds and loosened contaminants. Disinfect with 200-ppm chlorine for 1 h or with 100-ppm chlorine for several hours. Scrub tank bottom and sidewalls again. Rinse several times with clean freshwater and dry under the sun.

Earthen ponds – Drain the pond and then dry. Apply lime (0.5-1 ton/ha $CaCO_3$ or agricultural lime) and 20-ppm tea seed cake, or any of 600-ppm Roccal (benzalkonium chloride), Hyamine 1622 and Hyamine 3500 (quaternary compounds).

Disinfecting rearing water

Chlorination method — Chlorine is the cheapest disinfectant. One of the best and commonly used is calcium hypochlorite (powder form) or ordinary household bleach (Purex, Chlorox). Filter the water first. Chlorine loses its strength when exposed to air. It is reduced by organic matter (mud, slime, plant matter) and must be covered. Use 5 to 20-ppm chlorine for 12-24 h, then neutralize with sodium thiosulfate until residual chlorine becomes zero. Chlorinated, neutralized water must be used within 6 h as bacterial load increases after 12 h.

Ozonation method – Ozone (O_3-triatomic oxygen) is a more powerful oxidizing agent than hypochlorite. It can de-activate or destroy viruses and bacteria that might be transmitted through the water supply system. At 90-mg/L concentration and exposure for 20 min, ozone can control bacterial and viral fish pathogens in water supplies, although this level may not eliminate 100% of pathogens. Like chlorine, ozone is toxic to aquatic organisms. Oxygen (O_2) is a breakdown product of ozone, and oxidizing action may result in oxygen supersaturation or gas-bubble disease. The concentration of 0.005-ppm O_3 is the upper limit for continuous exposure. Ozonated water must be re-aerated before it is used.

Disinfecting materials

Materials like pails, brushes, scoop nets, secchi disk, glasswares, hose, etc. may be disinfected in between use in different culture facilities. Dip the materials in 400-ppm chlorine solution for a few seconds, and rinse thoroughly with clean water.

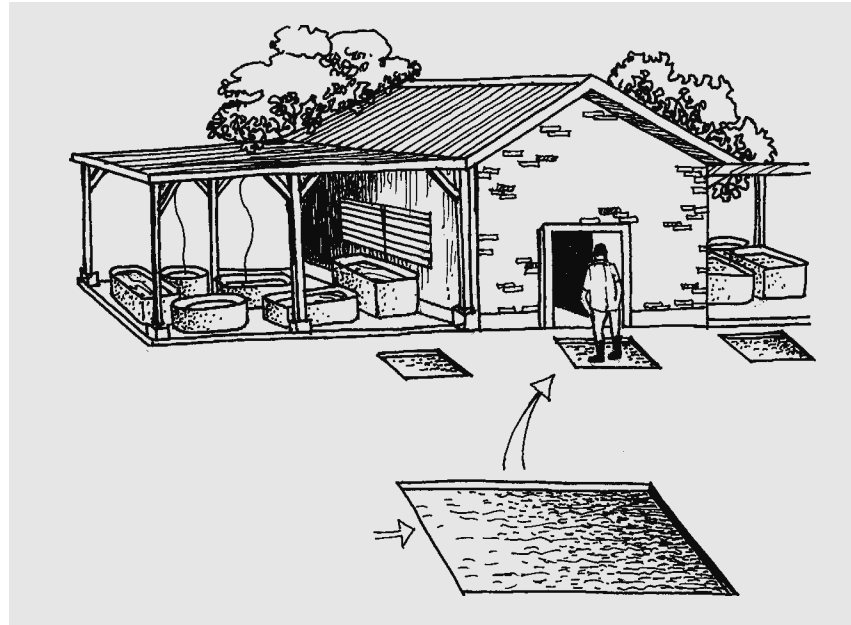

Figure 8-6. Disinfection rugs and trays at entrances of hatchery facilities

Disinfect footwear by placing 200-ppm chlorine or 3% Lysol solution in disinfecting rugs/trays at the entrance of aquaculture facility (Fig. 8-6). Wash rugs and change disinfectant regularly.

Disinfecting feeds

Artemia cysts – Cysts may be decapsulated in chlorine solution. Use 30 ppm chlorine or 10 ppm formalin, 1 h before hatching.

Disinfecting the hosts (especially *Penaeus monodon*)

Spawners – Disinfect with 5 ppm Treflan for 1 h or 50-100 ppm formalin for 30-60 min. Rinse spawners thoroughly in clean water.

Eggs – Disinfect with 20 ppm detergent for 2-4 h. Disinfection should be done at least 6 h before hatching. Rinse thoroughly and completely change water in hatching tank.

Larvae – Disinfect with 0.1 ppm Treflan (trifluralin) once every other day.

Chemotherapy

Chemotherapy involves the use of drugs or chemicals for treating infectious diseases. It is considered as the method of "last resort" in any disease control program.

Factors to consider before using chemicals:

- Tolerance of the host to the chemical – Tolerance of fish varies with age, size, species, and health condition. Younger or smaller fish are more sensitive than bigger or older ones. Some species are better able to tolerate chemicals than others are. Fish weakened by disease become less tolerant to stress and environmental fluctuations.

- Efficiency of the chemical – The choice of what chemical to use is based on differential toxicity, that is, the chemical must be lethal to the target micro-organism but harmless to the host. It is essential to know the properties of the chemical such as the active ingredient, solubility and application method. The chemical must not harm the environment.
- Restrictions on the use of chemicals to treat food fish – Use only chemicals that break down rapidly and are eliminated quickly from all fish tissues to avoid tissue residue problems. The chemical must not form toxic or carcinogenic products during cooking of the contaminated flesh.
- Consequences of drug resistance – The indiscriminate use of antibiotics may lead to the development of drug-resistant strains.
- Economics – Chemicals are expensive, and one should know the value of the stock and the cost of treatment to determine the benefits that may be derived from their use.

The methods of chemical treatment are as follows:

1. External methods – These are used to control ectoparasites and other micro-organisms outside the fish. They are employed to reduce or eliminate potential pathogens from tanks, ponds, and from other materials. The external method may either be topical or by immersion.

Topical

This is the direct and simplest method for treating wounds, skin ulcers and other localized infection. Immobilize the fish before taking it out of the water for treatment. Apply the drug directly on the infected area. The method is labor-intensive and should be used only for high-value fish.

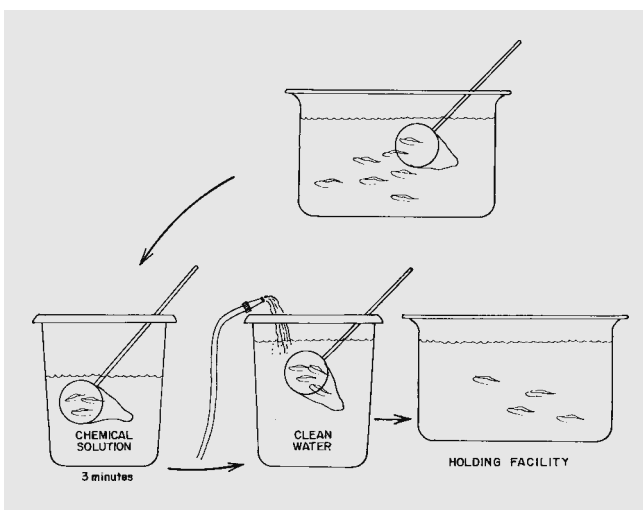

Figure 8-7. Dip method – fish are scooped out and suspended in the chemical solution for a few seconds to 3 min; rinse well with clean water and return to the clean/disinfected holding facility

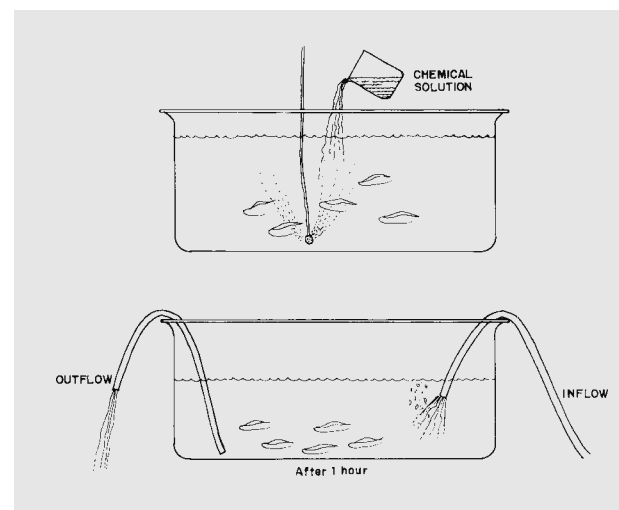

Figure 8-8. Short bath method – the chemical solution is added to the holding tank with fish, allowing the chemical/water mixture to remain with the fish for several minutes to 1 h; the solution is removed right after treatment and replaced with new, clean water

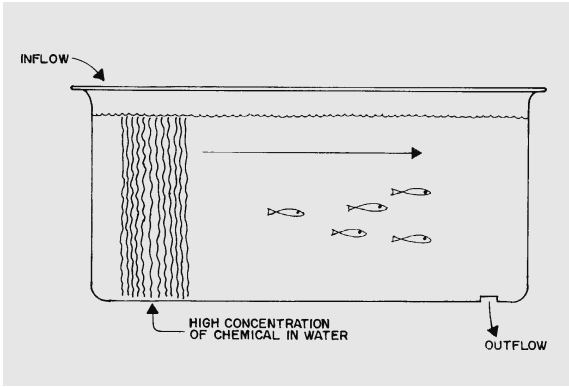

Figure 8-9. Flush method – the chemical solution is added in concentrated form at the inlet and allowed to pass through the water flow system and out the effluent pipe

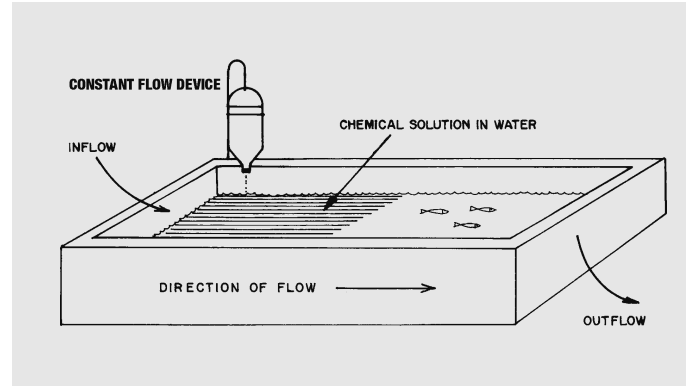

Figure 8-10. Flow through method – the chemical is added at a constant rate by a constant flow device; the chemical moves through and out of the container to be replaced by new clean water

Immersion

Dip. Place the fish in a scoop net and immerse in a high concentration of chemical solution for a specified time, usually from a few seconds to a few minutes. Rinse the fish immediately in clean water after treatment and return the fish to the clean/disinfected holding facility (Fig. 8-7).

Short bath. Add the chemical solution to the holding facility where the fish are to be treated, allowing the fish to remain in the chemical and water mixture for a designated time, usually a few hours or less (Fig. 8-8). After treatment, remove treated water immediately and replace with clean water.

Flush. Add a highly concentrated chemical solution at the water inlet and allow this to pass through the water flow system and out of the effluent pipe (Fig. 8-9).

Long bath. Treat the fish for a longer time, usually 12 h or more, in a chemical solution of low concentration.

Flow-through. Add the chemical at a constant rate through a metering device to give a consistent low concentration for the desired treatment time. The treated water moves through and out of the holding facility, and is replaced by new clean water (Fig. 8-10).

2. Systemic treatment – This is employed for treatment of systemic infections. Chemicals are added into the feed. The advantages of this method are that fewer chemicals are needed, environmental pollution is lessened and labor input is low. The disadvantages are the non-feeding of sick fish and that, since some drugs are not stable in moist diets, this would require introduction of more palatable components.

3. Parenteral treatment – This is the direct and most effective route of drug administration. Advantages are that accurate dosage can be administered and pollution of the environment is avoided. The disadvantages are that it is labor-intensive, it contributes to handling stress and it is good only for big and valuable stocks.

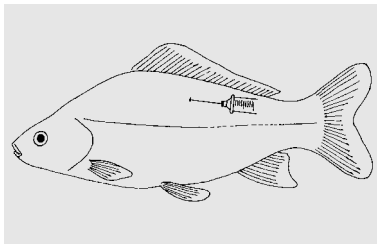

Figure 8-11. Intramuscular injection – needle is inserted into space posterior to the dorsal fin above the midline of the body

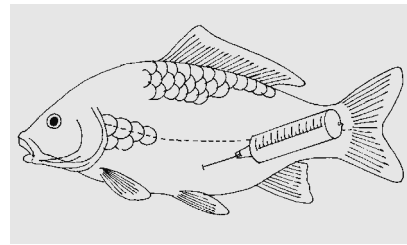

Figure 8-12. Intraperitoneal injection – needle is inserted into the visceral cavity or belly of the fish

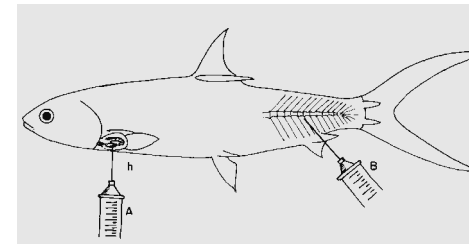

Figure 8-13. Intravenous injection – needle is inserted by (A) direct cardiac puncture or (B) through the caudal artery

Intramuscular. Insert the needle posterior to the dorsal fin above the midline of the body (Fig. 8-11). Absorption is slow (not very effective) or, sometimes, does not take place at all.

Intraperitoneal. This is the most common method of injection. Insert the needle into the visceral cavity or belly of the fish (Fig. 8-12). The drug must be highly absorbable and should be able to pass through either the intestinal wall or some other membrane to be absorbed into the fish system.

Intravenous. Insert needle by direct cardiac puncture, or through the caudal artery (Fig. 8-13). This results in rapid dispersal and is the most effective route for administering antibiotics. The only drawback is that this can be used only on large fish.

Disadvantages of Chemical Treatment

- If used in closed recirculating systems, chemicals may cause adverse effects or destroy the nitrification processes in biofilters.
- Treatment may have adverse effect on natural food. It may induce oxygen depletion during their degradation, or may destroy algal blooms whose decay then depletes the dissolved oxygen in the water. It may also inhibit photosynthetic production of oxygen.
- Chemicals may leave harmful residues in the host.
- Diseased animals do not eat medicated feeds.
- Baths usually do not result in therapeutic tissue level and may be ineffective against systemic infection.
- Antibiotic-resistant strains may develop.
- Some drugs have immunosuppresive effects.

Prevention of Drug Resistance

- Ensure correct diagnosis of the case.
- Use the prescribed dosage for a given period.
- Restrict use of drugs.
- Simultaneously administer two drugs that will not result in cross-resistance.
- Strictly observe implementation of the clearance/withdrawal period before the fish/shrimp can be harvested/consumed. In the tropics, this usually takes 2-3 weeks.

Principles of Bioassay

A biological assay is a procedure involving use of the responses of aquatic organisms to detect or measure the presence or effect of one or more substances, wastes, or environmental factors, alone or in combination.

Types of bioassay

Short-term. This type of bioassay reveals in relatively less time (usually 8 days or less) the relative toxicity of different toxicants to a selected test organism. It shows the relative sensitivity of various organisms to different conditions or variables like temperature and pH. It also determines the median lethal concentration (LC50), or the effective concentration values.

Intermediate. This bioassay is used when LC50 determination requires additional time (usually 8-90 days) for studies of the life stages of organisms with long life cycles, and to indicate toxicant concentrations for life cycle tests.

Long-term. This bioassay procedure is almost always a flow-through test. It determines the maximum allowable toxicant concentration, or safe concentration, for indicating water quality standards;

Methods of adding test solutions

Static. The test animals remain in the same test concentration for the duration of the test.

Renewal. This is a static test where the test animals are transferred to a fresh test solution of the same composition at periodic intervals, usually every 24 h.

Re-circulation. This static test involves the circulation of test solution through test chambers. The test solution may be treated by aeration, filtration, sterilization or other means to maintain water quality.

Flow-through. Measured quantities of dilution water and the stock toxicant solution are mixed and delivered periodically to the test containers to provide continuous flow-through of the test toxicant.

Test procedures

Criteria for selecting and preparing test animals:

Sensitivity to the materials under consideration;
Availability and abundance;
Recreational, economic and ecological importance;
Adaptability to laboratory conditions;
Suitability for bioassay tests;
Originating from a single common source;
Uniform in size and of the same stage of maturity;
Healthy, free from disease and parasites;
No previous exposure to heavy metals, pesticides, and other substances;
Acclimation to laboratory conditions for at least 10 days;
During acclimation, provide for daily feeding. Mortality should be less than 10% of the total population.

Experimental water. The water should not be polluted or contaminated with wastes from any source.

Experimental design. There should be a minimum of 5 test concentrations and control in at least duplicate containers.

Test concentrations. Express liquid waste concentrations as percent on a volume to volume basis. Express concentrations of non-aqueous wastes and of individual chemical composition by weight. Indicate whether the LC value is based on concentration of total material or active ingredient.

Test containers. Glass aquaria should be clean and of uniform size and shape. They should measure 15-30 cm deep and should be arranged at random in the testing area. If replicates are used, the series of test containers should be randomized separately.

Number of test animals or biomass. (1) There should be at least 10 animals per concentration. (2) Less than 10 animals may be used for the range finding test. (3) There should be a maximum of 1 g fish/ liter of water. (4) Distribute animals at random by adding one at a time to each container

Preparing test solutions. Test solutions must be freshly prepared. Also avoid unnecessary exposure to air and light.

Feeding. Do not feed for at least 2 days before the test. Do not feed during the entire experimental period for short-term tests (96 h or less).

Biological data and observations. Observe and record mortality at periodic intervals. The usual indicator for death is no movement, especially no gill movement in fish, and no reaction to gentle prodding. Remove dead organisms as soon as observed.

Report effects using terms like erratic swimming, loss of reflex, discoloration, changes in behavior, excessive mucus production, hyperventilation, opaque eyes, curved spine, and hemorrhaging.

Physical and chemical water quality. Measure temperature, salinity, hardness, alkalinity, pH, dissolved oxygen, ammonia and nitrite at the beginning of the test, and daily thereafter.

Calculation, analysis, and reporting of results. The recommended measures or indices of relative toxicity are 48 and 96 h LC50. Always compute for 95% confidence limits for LC50 and EC50 values.

The most widely used methods for calculating an LC50 and confidence limit are probit, logit, moving average and Lithcfield-Wilcoxon (1949). Other methods are straight line interpolation and the Reed-Muench method (1938).

Report the LC50 with specified exposure time and the confidence limits of LC50.

Provide descriptions of test organisms (species, source, size, weight), procedures for acclimation to test conditions and observations on behavior during the test). Describe also the source of experimental water and its characteristics, source and properties of tested material and concentrations of the test solutions. Indicate also the experimental temperature, test method, test conditions (type of container with volume and depth of solution, number of organisms), and the criterion of response.

SUMMARY

Disease prevention is a primary and cost-effective method in fish health management. It is more effective and economical than attempting to stop a disease that has already set in. The recommendations given above will greatly reduce the possibility of disease outbreaks.

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9

CHAPTER NINE

Immunity and biological methods of disease prevention and control

Jesus Manolo E. Almendras

Practically every multicellular organism, from invertebrates to vertebrates, is a potential host for various microbes. These microbes may spend some or all of their life cycle within, or upon, the bodies of their hosts. Any that gain entry to the tissues of their host may be rapidly distributed inside the host's body by its circulatory system. A host that does not have the means to protect itself from the entry and subsequent proliferation of microbes within its body can be rapidly overwhelmed. This chapter deals with the barriers that prevent microbial entry and the various internal defense mechanisms that are part of the host's arsenal in combating invading microbes. The latter part of this chapter discusses some of the biological methods of disease prevention and control.

The immune systems of fish and crustaceans have similarities and differences. Crustaceans are no different from other animals in that their defense system is largely based on the activities of the blood cells or hemocytes. These hemocytes, the crustacean equivalent of the vertebrate leucocytes, are capable of phagocytosis, encapsulation, nodule formation, and mediation of cytotoxicity. Another hallmark in crustacean immunity is the rapid sealing of wounds by blood coagulation to prevent loss of hemolymph and to immediately entrap invading microorganisms and arrest their dispersal in the body.

The fish immune system also has these non-specific mechanisms of defense but they can be differentiated from those of crustaceans as they have evolved an additional way of recognizing microbes. This recognition is the basis of what is commonly called the adaptive immune system or specific immunity. The adaptive system has two hallmarks that distinguish it from non-specific immunity. Firstly, recognition is performed by a receptor that exists in billions of different forms in an individual. This diversity endows the animal the ability to recognize any microorganism. Secondly, the adaptive system retains a memory of each particular microorganism to which it has been exposed. Memory allows the adaptive system to eliminate the same microorganism more effectively upon subsequent exposure.

The receptor that is responsible for these remarkable properties is the antigen receptor. These are found only in lymphocytes, which are found only in vertebrates. The substance or ligand, which the antigen receptor binds to, is called the antigen. Many substances that are foreign to the host can be an antigen, including proteins, polysaccharides and nucleic acids. These substances are usually components of the cell walls of microbes. There are two general catego-

ries of lymphocytes based on the antigen receptor they carry: T-lymphocytes and B-lymphocytes. The binding of antigens to their receptors triggers lymphocytes to become active in an immune response, a complex process usually referred to as lymphocyte activation. An activated B lymphocyte starts to manufacture large quantities of immunoglobulin molecules, which are then released into the blood. These soluble forms of immunoglobulin are commonly called antibodies.

THE FISH IMMUNE SYSTEM

Immunity in fish is mediated by two major systems: the innate or non-specific immune system and the adaptive or specific immune system. The innate immune system is thought to be of ancient origin and acts as the first line of defense against invading pathogens. It has no memory component and is active against a variety of microbial antigens. On the other hand, the adaptive immune system is present only in the vertebrates above the level of the agnathans. The most distinctive characteristics of the adaptive immune response are high specificity for microbial antigens and memory. Unlike the innate immune response, the adaptive response is not immediate since it would take time to synthesize specific antibodies against an invading antigen. Thus it constitutes the second but more specific line of defense. A summary of the components of the fish immune system is shown in Table 9 - 1.

Innate or Non-specific Immunity

Innate or non-specific immunity refers to various physical and cellular attributes that collectively represent the fish first lines of defense against infectious disease. These defense mechanisms come into play immediately or within hours of an antigen's or an invaders appearance in the body. The non-specific immune resistance includes the following levels:

Table 9 - 1. The major components of the immune response in fish

	Innate immune response	Adaptive immune response
Soluble Factors	Acute phase proteins Enzyme inhibitors Cell lytic enzymes Agglutinins	Antibodies
Cells	Phagocytes Natural killer cells	B – lymphocytes T – lymphocytes

Physical Barriers

The skin, including the scales in some fishes, and the mucous membranes of the digestive tract are physical barriers that protect the animal from harmful environmental agents and from invasion by microbes. Most of the fluids that are excreted or secreted onto epithelial surfaces, such as mucus and digestive juices, contain chemical components that are anti-microbial. Mucus is secreted by specialized goblet cells in the epidermis of fish and contains immunoglobulins (IgM). Mucus also contains precipitins, natural agglutinins, lysins, lysozyme, C-reactive protein and complement. However, the skin and other epithelial surfaces of fishes harbor a variety of microorganisms whose presence and numbers pose no threat to their health.

Soluble (Humoral) Barriers

The internal fluids (humors) of fish contain a number of substances that react with a variety of microbes to lyse, or coat them or to inhibit their growth. Among these substances are inhibitors of microbial growth (transferrin, lactoferrin, ceruloplasmin, metallothionein, cecropins, defensins, magainins), cell lytic enzymes (lysins, lysozyme, proteases), enzyme inhibitors, agglutinins and precipitins and interferons).

1. Acute Phase Proteins and Complement Factors

   The concentration of a number of protein types in the blood serum increases rapidly during an infection. These proteins are called *acute phase proteins.* One representative of the acute phase proteins is the *C-reactive protein* characterized by its ability to bind to the surface molecules of the cell wall of a wide variety of bacteria and fungi. When the C-reactive protein binds to the surface of bacteria, another group of proteins present in the blood, called *complement factors*, binds firmly with the immobilized bacteria and the whole complex of bacteria, C-reactive protein, and complement becomes more rapidly engulfed by phagocytes. This process of facilitating phagocytosis of bacteria by coating them with protein is called *opsonization*. In fish, C-reactive proteins are naturally present at a level that is 500 times higher than in mammals, a possible indication of the relative importance of the nonspecific resistance mechanism in fish.

   The complement factors of fish blood are also proteins. One of these proteins will react spontaneously with surface components of bacteria, notably the lipopolysaccharide (LPS) found on bacterial cell walls. The same complement factor also reacts with β-1,3 glucans which are structural components of bacterial and fungal cell walls. This reaction is called an activation because it elicits a sequence of reactions where one complement factor activates the next in a chain reaction that produces protein fragments with different properties. After activation, some of the complement factors are able to cause opsonization of bacteria, whereas others attain the ability to attract phagocytes.

   The activation of the complement system by external factors, such as LPS and β1,3 glucans is called the *alternative pathway* of activation. The complexes formed when antibodies react with antigens can also activate the

complement system. This is called the *classical pathway* of complement activation.

The different proteins in the complement system must act in combination in order to exert an antimicrobial effect. Moreover, lysozyme produced by phagocytic cells acts synergistically with the complement factors by its hydrolytic effect on the bacterial cell wall. Measurement of the level of complement factors and of lysozyme in the serum, and measurement of the phagocytic and bactericidal activity of phagocytes that operate in concert with these factors, provide ways to quantify the degree of non-specific resistance of an organism.

The C-reactive proteins, complement, and phagocytic cells constitute the most important elements of the non-specific immune system. Because this system exists with its same basic elements in all levels of the evolutionary system from marine invertebrates to warm-blooded animals, it is likely that it has its origin far back in the evolutionary process.

2. Transferrins and Lactoferrin

   Transferrin is a serum protein of 70 to 80 kDa molecular weight belonging to the group of acute phase proteins and present both in mammals and fish. Transferrin plays a possible role in delaying the start of microbial infections by binding the available iron and thereby depriving bacteria of an essential growth factor. The mucus secretions of mammals contain another closely related iron binding and antimicrobial protein called lactoferrin, but this has not yet been found in fish; lactoferrin does, however, enhance the disease resistance of trout when given in the feed.

3. Caeruloplasmins

   This is an acute phase protein present in fish that binds copper and other divalent metal ions. Caeruloplasmin acts as an oxidase that oxidizes divalent iron to ferric ions, which then bind to transferrin. By this reaction, ceruloplasmin contributes to depriving bacteria of available iron and divalent ions.

4. Metallothionein

   Metallothionein is a peptide rich in cysteine and with a high affinity for metal ions. Metallothionein has been found in several fish species. Its production is stimulated by metals in the environment and by endotoxins (LPS) from bacteria. It also deprives bacteria of essential metallic ions by sequestering them.

5. Enzyme Inhibitors

   Protease inhibitors that correspond to the mammalian α-2-macroglobulin are present in fish and are believed to play a role in retarding the invasion of pathogens or parasites. The other common groups of protease inhibitors are present in fish serum (inhibitors of serine proteinases, cysteine proteinases, aspastic proteases, metallo proteinases) and some of these belong to the acute phase reactants. Virulent pathogenic microorganisms secrete pro-

teolytic enzymes to digest and penetrate the tissues of their host. It is believed that the protease inhibitors of fish serum play a role in defense by neutralizing the proteolytic enzymes produced by these pathogens.

7. Cell Lytic Enzymes

   Lysozyme is a bacteriolytic enzyme occurring both in plants and animals. There are large variations in lysozyme activity in different fish species. Lysozyme is present in most tissues and secretions of fish, and the level seems to vary in relation to environmental conditions. Stress due to handling of fish and to pollutants reduces lysozyme activity in fish. Because lysozyme is produced in macrophages, the reduced level may be a reflection of a reduction of the macrophage function

   Fish serum contains molecules other than lysozyme that cause lysis of bacteria and probably have a function in the non-specific defense to infections. Fish mucus also contains a trypsin-like protease activity that is able to cause lysis of Gram-negative bacteria. This enzyme is produced in the mucus-secreting cells of fish and may function in concert with lysozyme and hemagglutinins in non-specific defense.

8. Agglutinins

   Fish serum, skin, and mucus contain factors that resemble immunoglobulins or antibodies in their ability to agglutinate and cause lysis of foreign cells and bacteria. These agglutinins are non-specific and they correspond to lectins of invertebrates in their ability to recognize and bind to single sugars on the bacterial surface and mediate phagocytic reactions

Cellular Barriers of the Innate Immune System

1. Inflammation

   Inflammation is the observable condition that accompanies damage to the body. It is a localized response to tissue injury and to invading microorganisms, characterized by infiltration by granulocytes and macrophages, removal of dead cells and foreign cell and debris, followed by tissue repair. Inflammation confers protection by walling off an infected area from the rest of the body. This type of response in fish has been reported against bacterial, viral, fungal, protozoal and parasitic infections. In the higher vertebrates especially in mammals, inflammation involves mast cell degranulation and the release of vasoactive substances. These cause vasodilation, which increase blood flow and vascular permeability, and adhesiveness of vascular endothelial cells for phagocytic blood cells.

2. Natural Cytotoxic Cells

   Some population of cells in fish display a non-induced and non-specific toxicity to foreign cells. The non-specific cytotoxic cells in fish are equivalent to the natural killer cells of mammals. They differ from their mammalian counterparts by being able to destroy a wider range of foreign cells, and they can even destroy multicellular parasites that attack fish.

3. Phagocytosis in Fish

In fish, macrophages, monocytes and granulocytes are phagocytic and in some species neutrophils are also phagocytic. Intracellular killing by teleost macrophages is similar but slower than found in mammals. These killing activities include lysosomal enzymes, alkaline and acid phosphatases and peroxidases. Activated macrophages produce oxygen metabolites such as super oxide anion in a process known as the respiratory burst. These oxygen radicals are bactericidal.

Adaptive or Specific Immunity

The concept of specific immunity includes three important components: the ability to recognize and respond selectively; the ability to recognize and respond preferentially to foreign substances and the ability to respond better on repeated exposure to them (memory).

Antigens

The specific substances that trigger an immune response are called antigens. Most antigenic substances are large molecules like proteins, polysaccharides and nucleic acids, but usually the immune system only recognizes and responds to a small part of these large molecules, called the antigenic determinant or hapten. So, each antigenic substance can have many different haptens in one large molecule. Small foreign molecules can also act as haptens when attached to a larger molecule.

Cells

The two types of cell that are involved in specific immune responses are lymphocytes (the B and T lymphocytes) and antigen-presenting cells (APCs), which include macrophages (Figure 9 -1).

Receptors

Lymphocytes, but not APCs, have membrane receptors for specific antigens. These receptors are proteins that specifically recognize and binds to an antigen. They are the means all lymphocytes use to recognize antigens and some use to respond to them (Figure 9 -1).

Humoral vs Cellular Immunity

There are two different forms of specific immunity: humoral (or antibody-mediated) and cellular (or cell-mediated). Cellular immunity acts by direct cell-to-cell contact to protect the body against viruses that have infected its own cells and against tumor cells. Humoral immunity acts by the secretion of soluble proteins (antibodies or immunoglobulins) that circulate in the blood and lymph where they can combine with antigens and neutralise them.

Humoral immunity

Active and Passive

Humoral (antibody-mediated) immunity can be either active or passive. Active immunity occurs when the antibodies are made within the body's immune

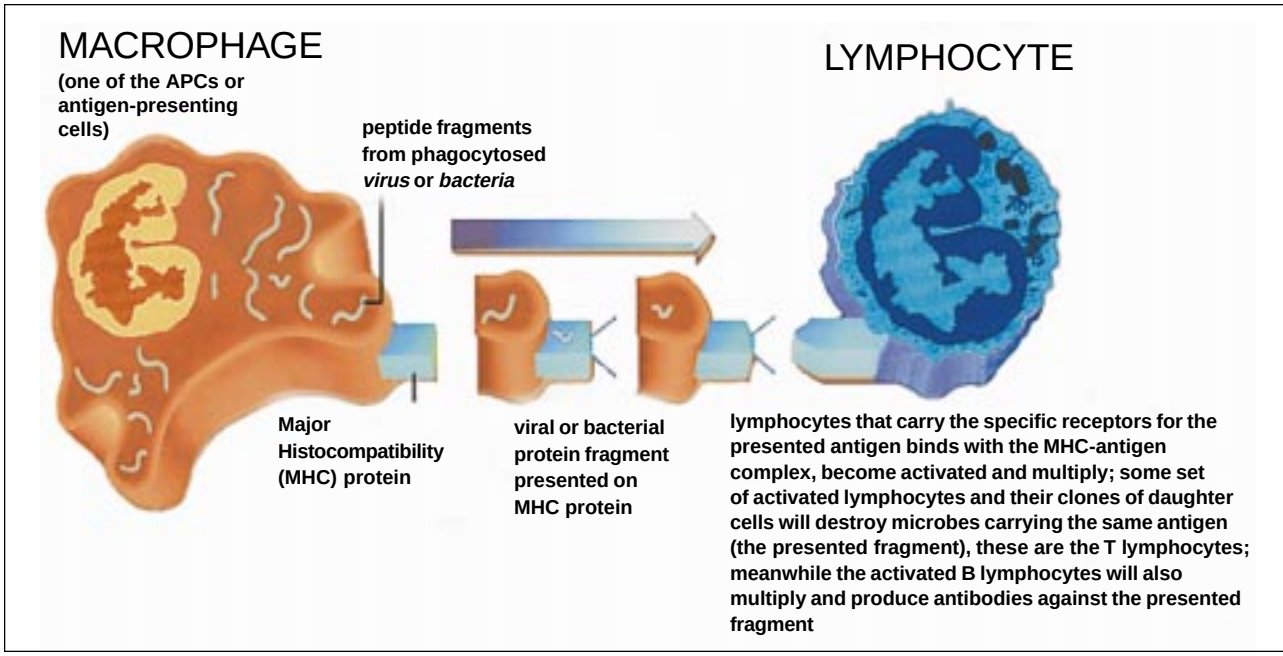

Figure 9-1. The process of presentation of a foreign peptide or protein fragment from an invading microbe by the MHC protein of a macrophage and the binding and activation of specific lymphocytes that carry the corresponding receptor to the presented fragment

system. It is passive when the antibodies come from outside the body. An example of this is the colostrum of mammalian milk, for a few months after birth a mammalian infant is naturally protected by passive immunity gained through antibodies secreted in its mother's milk, this is also true in some fish eggs and larvae that contain some antibodies produced by the parent fish. Antibodies can be extracted from plasma and injected into a patient as an artificial but temporary means of providing immediate protection against certain antigens.

Antibodies (Immunoglobulins - Ig)

Antibodies are Y-shaped proteins formed from two pairs of polypeptide chains (two heavy and two light chains) held together covalently by disulfide bonds. Each antibody can bind selectively to one type of antigen. But each arm of the Y has a binding site, so each antibody molecule can bind to two molecules of the same antigen. The stem of the Y can be recognized by other cells of the body, so it helps determine what happens to an antigen-antibody complex.

Antibody Diversity

The binding sites of an antibody are specific and highly selective for a single antigen. The body is capable of making an antibody that can combine with any conceivable antigen. The genetic mechanisms that allow this are complicated, but very fascinating. In addition, any particular antibody can occur as different classes of immunoglobulin (IgG, IgM, IgE, IgA, IgD), which are effective in different circumstances. They differ in the kind of protein that forms the stem of the Y (parts of the pair of heavy chains), although they have the same anti-

genic specificity. The cells (activated lymphocytes) that make a specific antibody can switch from making one class to making a different class, but always of the same antigen specificity.

Antibody Production

Plasma Cells

Plasma cells are the effector cells of humoral immunity. They synthesize and secrete antibodies (IgM and IgG). Plasma cells are B lymphocytes that have been activated by interaction with their matching antigen. The antigen receptors on the membranes of unstimulated B lymphocy'tes are IgM and IgD class antibodies that have the same specificity as the IgG that they will eventually produce as plasma cells. Plasma cells are mostly located in lymphoid organs (but not in the thymus) but are sometimes released into the blood.

The purpose of B lymphocyte stimulation is to form enough plasma cells to produce useable amounts of antibodies. It is a complex process. Besides the B lymphocytes themselves, it involves accessory cells (e.g. macrophages) and a specific class of T lymphocytes (helper cells). It also depends on the presence of special membrane proteins (major histo-compatibility or MHC proteins) on the surface of these cells and the release of different paracrine and autocrine chemical messengers (cytokines) by them.

There are two classes of MHC proteins, MHC-I and MHC-II. All cells (except RBC's) have MHC-I proteins. Only a few cell types have MHC-II proteins. These include macrophages and other accessory cells (including B lymphocytes) that can function as Antigen Presenting Cells (or APC's presenting antigens to lymphocytes).

Accessory cells (such as APC's) ingest antigens and partially digest them. Fragments of the antigens (containing the haptens) are combined with MHC-II proteins and then together they are inserted into the plasma membrane. Helper T lymphocytes can recognise the MHC-I protein-hapten complexes if they also have receptors that can bind to the hapten. Recognition (or when the receptor on the lymphocyte is a complimentary match to the presented antigen) triggers the release of several kinds of cytokine (interleukins). The interplay between cell-to-cell contact and chemical co-stimulation is what finally activates the B cell (or B lymphocyte).

The process of B cell stimulation results in mitosis of both the helper T cell and the B cell, with the formation of a clonal population of each. This results in a very large number of cells, all producing identical antibodies specifically against the presented antigen (Figure 9 – 1). Some of the B cells are further activated to become plasma cells, which provides a large enough group of cells to produce enough antibody to combat the antigen while some cells become the long-lived memory cells (see text below and captions in Figure 9 – 1).

Immunologic Memory

Primary and Secondary Responses

The first time the body is exposed to a particular antigen, the resulting immune response is mild and brief. The second time that same antigen is encountered,

however, the response is strong and long-lasting. This is an important aspect of the specific immune response. How strong and long-lasting the secondary response is can vary from antigen to antigen. Immunization against specific pathogens is aimed at establishing immunologic memory through repeated, controlled exposures to their antigens (with little or no chance of being infected).

Memory B and T Lymphocytes

Some of the activated B cells that are formed as part of the primary immune response become short-lived plasma cells that produce antibodies (IgM and IgG). Others persist as memory B lymphocytes. These cells are able to multiply and transform rapidly into plasma cells during a secondary response, when they produce some IgM and lots of IgG. In the activation of T lymphocytes (both helper and cytotoxic T cells), memory T cells are also formed.

Cellular Immunity

Cellular immunity and humoral immunity have several similar features . T cells have membrane proteins that bind only to a specific antigen, just as B cells do. T cells rely on displaying fragments of antigens combined with their MHC proteins. T cells must be co-stimulated by interacting with other cells that present the same antigen-MHC combination. An antigenic challenge causes the co-stimulated cells to release cytokines that stimulate single T cells to divide to form clones of helper T cells and cytotoxic T cells.

Cytotoxic T cells are the effector cells of cellular immunity. They can cause the death of antigen-bearing cells in several ways, but all require that they have close contact. This explains why T lymphocytes circulate through the blood, connective tissues and lymph - they are searching out foreign or abnormal cells. This immune surveillance is aimed at cells infected by viruses, bacteria or parasites and at tumor cells. Cytotoxic T cells also attack foreign cells that are present in blood transfusions and tissue transplants. People with organ transplants (such as heart transplants) must take immunosuppressive drugs to suppress the cytotoxic T cells and allow the transplanted organs to survive.

THE CRUSTACEAN IMMUNE SYSTEM

The crustacean immune system lacks the immune memory characteristic of vertebrates and have to rely on the innate or non-specific response. However, crustaceans are no different from the vertebrates in that their immune defense is largely brought about by the activities of specialized blood cells or hemocytes. These crustacean hemocytes carry out phagocytosis, encapsulation, produce antimicrobial substances to remove or neutralize foreign particles and infectious agents.

Hemocyte types

Based on their morphological appearance and their staining properties, different hemocyte types can be distinguished in crustacean blood. However, these hemocyte types do not fall into the same groups as those found in fish and other vertebrates. The three types of hemocytes that can be isolated from crustacean hemolymph are the *hyaline*, the *semigranular*, and the *granular* (see Table 9 –2 for summary of their functions).

Table 9 – 2 . Crustacean hemocyte types and their known biological functions

Hemocyte type	Functions			
	Phagocytosis	Encapsulation	Cytotoxicity	ProPO Activating System
Hyaline	Yes	No	No data	No
Semigranular	Limited	Yes	Yes	Yes
Granular	No	Very limited	Yes	Yes

The hyaline hemocytes have phagocytic ability. This cell type, which lacks granules, is found in decapod crustaceans, but apparently the relative number of this cell type vary considerably among species.

The semigranular hemocytes are characterized by the presence of a number of small granules in their cytoplasm making them resemble the granulocytes of vertebrates. These cells respond to microbial cell wall polysaccharides such as bacterial lipopolysaccharides (LPS) and the β-1,3 glucans of fungi. They also have the ability to encapsulate foreign particles.

The granular hemocytes are characterized by the presence of large vesicles or granules in their cytoplasm. This suggests a role in the production, storage and secretion of antimicrobial compounds. They do not have phagocytic activity and their ability to encapsulate foreign particles is limited. The primary role of granular hemocytes is to store prophenol oxidase, which is key compound in crustacean defense reactions. These cells can be induced to unload and activate the prophenol oxidase by the presence of B-1,3 glucans, peptidoglycans and LPS from microorganisms. Once activated, the phenol oxidase catalyzes the oxidation of phenols to semiquinones and quinones that are highly toxic to microbes due to their high reactivity.

Defense Mechanisms in Crustaceans

Phagocytosis

Phagocytic cells are found throughout the animal kingdom. In lower invertebrates they also serve a nutritive function and in higher phyla they become more specialized by assuming a defensive role against microbial infections. A

microorganism that has penetrated the exoskeleton and enters the tissues or the blood is immediately attacked by phagocytic cells that are specialized to engulf and digest particulate matter. In crustaceans, these are the hyaline hemocytes. Their primary function is to clear the body of foreign particles including virus, bacteria and fungal cells. Objects too large to be phagocytosed by one cell are trapped inside aggregates of hemocytes.

Nodule Formation and Encapsulation

When crustaceans are invaded by a large number of microorganisms that exceed the capacity of phagocytic cells, nodule formation or cell clumping occurs. The microorganisms become entrapped in several layers of hemocytes, and generally the nodule become heavily melanized because of the host's phenoloxidase activity.

When a parasite is too large to become engulfed by phagocytosis several hemocytes will then collaborate by sealing off the foreign particle from circulation. This process is known as encapsulation. The semigranular hemocytes are the first cell to react to foreign particles and to encapsulate any invading intruder. However, little is known about the mechanisms by which foreign particles or microbes are dealt with after being engulfed or encapsulated by hemocytes.

Cytotoxicity

Warm-blooded animals (higher vertebrates) produce a specialized white blood cell capable of killing tumor cells and cells infected by virus. Such cells are called natural killer cells. Among the white blood cells in the hemolymph of crustaceans there are populations of specialized hemocytes that, like the natural killer cells in mammals, have the ability to kill foreign cells, tumor cells and non-tumor target cells.

Lectins

Agglutinating substances or lectins are present in the blood of a number of different crustaceans. Lectins are proteins or glycoproteins that have the ability to recognize and bind to the carbohydrates on the bacterial or fungal surfaces. Lectins do not have catalytic or enzymatic activity, their action is simply to immobilize or agglutinate microorganisms and then mediate the binding between hemocyte surfaces and the microorganisms (or other foreign bodies) and thus function as an opsonin.

Antimicrobial Proteins or Peptides

The immune systems of arthropods also rely on the production of proteins and peptides that possess antimicrobial activity against a wide range of microorganisms. However, in crustaceans the presence and characterization of these antimicrobial peptides has been poorly studied until now. Recently, hemocytic proteins have been isolated in the crab, *Carcinus maenas* and a 6.5kDa antimicrobial peptide has been characterized. In the penaeids, three antimicrobial peptides have been isolated from the hemocytes and plasma of *Penaeus vanamei*. They have been fully characterized and their cDNA cloned. Based on

their biochemical and structural features, the three peptides do not belong to any group of peptides that have been hitherto described. The peptides were named penaeidins after the genus *Penaeus*. Studies on the role of the penaeidins in the immune system are still continuing, as are studies on the search and characterization of other antimicrobial peptides in shrimp.

The Clotting Reaction

Since crustaceans have an open circulatory system, wounds must be sealed immediately to stop blood loss and prevent the entry and distribution of microbes within the body. In the shrimp the clotting process requires the presence of plasma proteins and cellular components. The key plasma protein that constitutes the clot has been named clotting protein or CP. It appears to be present in relatively high concentration in the hemolymph. The clotting reaction in crustaceans differs from that of vertebrates because aside from tissue damage as a trigger for the clotting cascade, the presence of microbial LPS is also a stimulus for the release of transglutaminase from hyaline cells that triggers the clotting process.

The Phenol Oxidase System

Parasites and microbes can gain entry into a crustacean body through wounds or as contaminants in the food. Some pathogens like the fungi penetrate the exoskeleton by secreting proteases and exerting mechanical forces. The response to this invasion can often be seen as dark spots in the cuticle and the intruders will become brown-black. The cause of this is melanin which is one of the end products of the phenoloxidase system. The enzyme responsible for the formation of melanin is phenoloxidase (PO). This enzyme (PO) catalyzes the oxidation of phenols to quinines that subsequently polymerize into melanin. During the formation of melanin, transient oxidation products are also formed which are highly reactive and toxic to microorganisms. The phenol oxidase system is thus an important component of the crustacean immune system.

A crucially significant feature of the phenol oxidase system is that it is able to identify a real infection and it can only be switched on by signals that are uniquely associated with the physical presence of pathogens. Crustaceans use the lipopolysaccharides LPS and the β-1,3 glucan molecules, which are components of the cell walls of microbes, as the specific signals to activate the phenol oxidase system. Crustacean hemolymph contains specialized binding proteins that seeks out and binds to the LPS and glucans of microbes. Once these binding proteins have reacted with their target LPS and glucans, they then bind to a specific receptor on the hemocytes (both semigranular and granular) and induce degranulation and the release of the prophenol oxidase, which can be converted from its proform into the active enzyme (phenol oxidase) again upon contact with microbial LPS and glucans. These specific binding proteins, whose structures have already been elucidated, can also act as opsonins that stimulate phagocytosis. Thus the crustacean phenol oxidase system is exquisitely designed to be specific against microbes and to avoid metabolically costly and harmful false alarms.

IMMUNOSUPPRESSION

A distinct subset of T lymphocytes, called suppressor cells, exists in the immune system to turn down antigen-driven responses, and as a mechanism to maintain tolerance to self-antigens in the periphery. Thus the immune system has a self-regulating mechanism to modulate its reactions especially to cells and tissues of its own. However, there are many situations where the suppression of the immune system is unwanted and may eventually lead to disease.

A number of internal and external factors exist that can cause this suppression:

Stress Stress can have marked effects on the health of fishes. Stress can come in many forms such nutritional stress due to improper diet and feeding schemes; environmental stress brought about by poor water quality and physical stress attendant to handling, crowding or confinement. These types of stress can sometimes be unavoidable in intensive fish farming. Prolonged exposure to stress or even very brief stressful experiences can depress certain aspects of the cellular and humoral immune systems consequently lowering resistance to pathogens. Once stress is experienced, a cascade of neuroendocrine events follows that generally leads to elevation of the steroid hormone, cortisol, in circulation. This hormone and other stress hormones (e.g. catecholamines) can reduce the number of circulating leukocytes and antibody-producing cells, and depress macrophage activity and distribution of leukocytes in to various body compartments.

Metals Aluminum, arsenic, cadmium, chromium, copper, lead, mercury, nickel and zinc are heavy metals that have been shown to lower the non-specific and specific immune response in fish making them more susceptible to various viral and bacterial diseases.

Aromatic Hydrocarbons Polychlorinated biphenyls (PCBs) have been reported to have modulating effects on the immune response in fish ranging from no effect reduction of antibody producing cells and increased susceptibility to disease. Chlorinated dioxin (TCDD) partially suppressed the mitogenic response in trout. It appears that fishes may not be as sensitive to halogenated aromatic hydrocarbons as do the higher vertebrates, like mice, with regard to their humoral immune system.

Pesticides Endrin, malathion, methyl bromide, triclorphon, DDT, Bayluscide and tributylin have immunosuppressive properties ranging from reduced lymphocyte number and phagocyte activity to necrosis of the thymus.

Drugs Oxytetraxycline, the antibiotic most used by fish culturist in treatment of bacterial disease, has been long known to be immunosuppressive in fish, reducing

the numbers of antibody-producing cells. Regardless of the mode of administration (injection, feeding or bath) an immunosuppressive effect is evident. However, oxolinic acid, a more recent addition to the list of drugs for treating fish bacterial disease, was found to not have immunosuppressive properties when used at the therapeutic levels recommended.

BIOLOGICAL CONTROL

At the turn of this century, the concept and practice of biological control was developed by agriculturists to control insect pests. In agriculture, biological control is generally defined as the use of a specially chosen living organism to control a particular insect pest. It is basically the use of one chosen organism to control another organism. This chosen organism might be a predator, parasite or disease, which will attack the harmful insect. It is a form of manipulating nature to increase a desired effect. In agriculture, a complete biological control program may range from choosing a pesticide, which will be least harmful to beneficial insects, to raising and releasing one insect to have it attack another, almost like a "living insecticide".

The scope of the concept of biological control was later expanded to include all kinds of pests. Such as the use of different species of carps to control the proliferation of water lilies and other plant pests that choke ponds and rivers. More recently, the concept of biological control was further expanded to include all forms of biological manipulation of the host, the environment and the pest (or pathogen) to minimize or control infestation or infection. It is this expanded scope of biological control that we will discuss here and we will focus on the use of biological control in preventing, minimizing or eradicating disease in aquaculture stocks.

Avoidance of Pathogens

Aquaculture facilities that use intensive techniques for production often provide an environment for the cultured organism that is unnatural and stressful. A primary factor is crowding or over-crowding. Under this condition the fish or the cultured organism has to compete with each other for space and dissolved oxygen and are confronted with the stress of exposure to increased metabolites such as ammonia, carbon dioxide and dissolve or suspended organic matter. It is not surprising that disease outbreaks often occur in these facilities. An aquaculturist's main concern then is to eliminate all possible sources of these disease agents.

Pathogen-free water supply

A pathogen-free water supply is obtained through the sterilization of fresh water or seawater. Sterilization of in-coming seawater may control the number of potentially harmful microorganisms entering the aquaculture systems but not necessarily totally eradicate these microorganisms in the water. The most commonly employed methods for controlling the presence of these microorganisms

are by filtration, ultraviolet light treatment, ozonation and chlorination.

1. Filtration

   Ultrafiltration of water supplies through sterile 0.2µm membrane filters has the advantage of having none of the detrimental effects associated with the other methods of water sterilization. However, it could be a slightly more expensive method than the other three and that absolute retention of bacteria (e.g. 100%) will not necessarily be achieved.

2. Ultraviolet light

   Ultraviolet light at a wavelength of 254nm or within the range 240 to 280nm disinfects seawater but not sterilizes it. It reduces the reproductive capacity of bacteria and fungi and is therefore bacteriostatic (or fungistatic) rather than bactericidal. Organic matter in seawater may also be oxidized depending on the ultraviolet energy emitted. The efficiency of ultraviolet light treatment is dependent upon the amount of particulate material suspended in the water and of the presence of natural pigments seawater. To improve efficiency, it is advisable to reduce particulate matter content by mechanical filtration prior to UV light exposure.

3. Ozone treatment

   Ozone is a highly oxidizing form of oxygen, if used with caution; it can be a powerful means of improving seawater quality. Ozone dissociates rapidly in seawater to provide a highly active oxygen atom. Ozone will disinfect and sterilize seawater, oxidize organic material and oxidize toxic nitrite to less toxic nitrate. However, ozone is highly toxic to both man and the organism being cultured. It is also highly corrosive to aquaculture equipment, be it metal or plastic. The effective dosage of ozone can be affected by factors that also consume ozone such as: chemical oxygen demand, salinity, dissolved substances, and microbial and plankton densities.

   The equipment manufacturers provide guidance on the use of ozonators and UV light cartridges.

4. Chlorination

   Chlorination, although less effective than ozonation, is the more popular form of seawater sterilization in aquaculture facilities in Southeast Asia. This could be due to the fact that chlorination does not involve special equipment. However, it shares many of the disadvantages of ozone in that it is toxic to both man and the cultured organisms, corrosive, and may form toxic stable complexes with organic compounds (e.g. chloramines). Chlorine as calcium or sodium hypochlorite is commonly used in hatcheries in south Asia.

Pathogen-free diets

Most manufacturers of artificial diets claim that their products are pathogen-free. In most cases the preparation of commercial feeds involves high temperatures of about 120°C for short periods that should be enough to pasteurize if not sterilize the feed.

On the other hand, the feeding of live food may present a different set of problems in that some germicides that are used to disinfect live feed could also be toxic to the live food organism. In Japan, live foods are managed under sanitary conditions to prevent intestinal infections of larval fish and shellfish. Bath treatments with a nitrofuran derivative, sodium nifurstyrenate, in live diets, such as rotifers and brine shrimp, are effective approaches to decreasing the number of bacteria in these diets.

Specific-pathogen-free (SPF) stocks

SPF animals are defined as "animals that are free of specified microorganisms and parasites but not necessarily free of others." Although specific pathogen free technology has been practiced in agriculture for many decades it has only been recently applied in aquaculture.

In prawn farming, some pathogens causing epizootics during the grow-out phase have been traced to be vertically transmitted from wild broodstock that are carriers of diseases. The vertical transmission of pathogens from mother to larvae is a continuous threat to production. In Taiwan, the screening of wild broodstock and the production of captive reared broodstock, which are certified SPF with regards to white spot syndrome virus (WSSV), have resulted in grow-out cycles free of WSSV epizootics. Although the use of SPF broodstock does not result in disease resistant or even disease tolerant stock, it is proving to be one effective managerial control measure, which minimizes the likelihood of epizootics due to an identifiable pathogen.

Disease inspection, quarantine, and international regulations

A fish health inspection is a procedure by which a sample of fish collected from a defined fish population and examined for the presence of certain specific pathogens. Knowledge of the presence of pathogens can be used to prevent the introduction of serious fish diseases into areas where they do not presently occur and to better manage those diseases.

A number of serious diseases are caused by organisms that can survive only for very short periods of time outside of the fish they infect. These organisms are called *obligate pathogens*. Fish health inspections are conducted to detect this obligate pathogen group because one of the most likely methods by which these are spread to new areas is through infected fish. Another broad group of pathogens are called *facultative pathogens*. They are commonly found in all aquatic environments and may cause disease only when the host fish is stressed. Fish health inspections are not typically conducted to detect organisms in the facultative group.

Quarantine is defined as the holding or rearing of animals under conditions that prevent their escape or the escape of a disease agent. Quarantine can provide a useful environment for "filtering out" disease in new stocks, especially if cures are available. Unfortunately post-treatment checks of individuals in large aquatic animal populations are generally not practical, and few cures are known for important fish and shrimp diseases. Quarantines are sometimes used as a means to avoid a particular disease agent. Such a concept often emerges as a regulatory effort to establish "disease free" or "pathogen free"

status of imports. While it is true that the quarantine technique is a means for disease avoidance, there is a great deal of difference between the presence of disease and the presence of pathogens.

A number of international organizations have been developing guidelines or codes of practice for the introduction and transfer of species. These guidelines establish specific diagnostic techniques for pathogens, define sanitary regulations of the individual countries, and develop health certificates for facilities to accompany shipments and prohibit the international transfer of aquatic animals that are not accompanied by these certificates. The organizations that have developed protocols include the Office International des Epizooties (OIE), the European Inland Fisheries Advisory Commission (EIFAC), and the International Council for the Exploration of the Sea (ICES). The ICES code of practice details how the species approved for importation are to be handled, the following are the steps of the protocol: 1) imported stocks are examined for potential pathogen or parasites, 2) stocks that are pathogen-free are grown into broodstock in an approved quarantine facility where they are regularly examined for pathogens, 3) if no pathogens are detected, the first generation offspring are released to the farmer, the original imported stock never leaves the quarantine site and disease studies are continued on the transplanted individuals, and 4) to eliminate the need for further importations of this species, it is recommended that F_1 individuals be used to establish a local broodstock.

Biology of the pathogen

The control of disease in an aquatic environment is particularly unique because water, as a universal solvent, makes prevention and control of physical, chemical and biological contamination of water and water sources much more difficult compared to land-based agriculture. Aside from the water itself, aquatic animals in all stages of their life cycles are carriers and reservoirs of disease.

Knowledge on the biology of the pathogen is one tool in breaking the cycle of infection and transmission. In shrimp hatcheries, for example, there is evidence that the virulence of facultative pathogens (e.g. *Vibrio harveyi*) increases from cycle to cycle. Successive passages from host to host provide a mechanism where the characteristic of virulence is selected. In order to limit the spread, adaptation and selection of these virulent strains, it is advisable to separate each group of larvae in time, (such as by batch culture, or by giving rest periods to the whole facility) and to isolate each group in space (by giving extra distance between tanks or using enclosures in some tanks). Any vector of contamination (e.g. water, workers, equipment and the fish themselves) must be controlled continuously to prevent vertical and horizontal transmission of pathogens. Another example is in the use of antibiotics in hatcheries. The use or overuse of antibiotics in hatcheries and farms may encourage outbreaks of oomycetes (*Lagenidium*, *Sirolpidium* and *Haliphthoros*) by removing the competitive bacterial microflora allowing these organisms to proliferate. An additional example where knowledge of the physiology of the pathogen can be put to good use is in cases of vibriosis. The *Vibrio* species that infect marine fish and shellfish are not tolerant to freshwater or very low salinities. If the infected cultured stocks happens to be euryhaline like the sea bass then the salinity of

the rearing water can be lowered gradually even down to zero parts per thousand salinity to eliminate the pathogen.

The Role of Stress in Disease

Because of the high stocking densities used in most aquaculture operations and the scarcity of water supplies free of aquatic microorganisms, a normally functioning immune system is absolutely essential to the health and physiological balance of the fish being cultured. The crowded conditions may increase initial susceptibility to infections and facilitate the horizontal or fish to fish transmission of pathogens when infectious disease outbreaks do occur.

Stress is defined as physical, chemical or biological factors that cause bodily reactions that may contribute to disease and death. Many potential fish disease pathogens are continually present in the water, soil, and air or in the fish themselves and outbreaks of clinical disease usually occur only when the fish is under some form of stress.

Aquaculture practices that increase stress are:

1. High stocking densities and poor water quality
2. Injury during handling (e.g. chasing, netting, sorting and shipping)
3. Improper nutrition
4. Poor sanitation

Some managerial practices that may help prevent stress:

1. Water quality
    - Do not exceed the carrying capacity of ponds and tanks.
    - Regularly monitor water quality parameters.
    - Prevent the accumulation of organic debris, nitrogenous wastes, carbon dioxide and hydrogen sulfide.
    - Maintain the appropriate or optimal pH, alkalinity, temperature and salinity for the species.
2. Handling and transporting
    - Exercise speed and gentleness when handling fish.
    - Use knitted mesh nets rather than knotted nets or better still, use smaller mesh size nets rather than larger mesh to reduce scale loss or entanglement of fins and finrays during capture.
    - Minimize the number of times that fish are handled or lifted from water and if possible use anesthetics to slightly sedate the fish.
    - Harvest, handle and transport fish at the point of their life cycle when they are least susceptible to stress, and in warm areas handle fish only during cooler periods of the day and add ice to the transport water to decrease fish metabolism and increase oxygen solubility.
    - Maintain high levels of oxygen as this is critical for the rapid recovery of fish from the struggle of capture and handling; for freshwater fish add salt at 0.3 to 1.0 percent in the transport water to minimize osmotic stress and bacterial infection.

3. Nutrition

   - Feed high quality diets that will meet the specific nutritional requirements of the species as different species have different levels of requirements for fatty acids, amino acids etc. and feeds that do not meet these needs are simply metabolized leading to increase excretion of wastes instead of being used for growth.
   - Use proper feeding rates and feeding schedules.
   - Store feeds in a cool dry place to preserve nutrients and prevent the growth of toxin producing fungi.

4. Sanitation

   - Quarantine all new fish.
   - Make sure that water supplies are not contaminated from the source.
   - Immediately remove all dead fish and dispose them properly to prevent the spread of diseases.
   - Observe good sanitation processes by disinfecting containers, nets and equipment to minimize transmission of parasites and disease from one population to another.

Improvements in Host Resistance

Dietary Enhancement

The resistance of fish to diseases involves a complex array of mechanisms that include maintenance of epithelial integrity and mucus coat, non-specific cellular factors such as phagocytosis by leukocytes, non-specific humoral factors such as lysozyme, complement, and transferrin, and specific humoral and cellular immunity. A variety of nutritional components can influence the incidence and severity of a number of infectious diseases. Some micronutrients that are known to enhance disease resistance include vitamins C, B_6, E, and A, and the minerals iron and fluoride. The role of the macronutrients (protein, lipid, and carbohydrate) in disease resistance has not yet been clearly defined. There are evidences that certain fatty acids may have essential roles in disease resistance.

Artificial diets that produce the best growth performance may not necessarily produce the optimal immune status. In marginal deficiencies, the fish continue to grow, appear healthy, and show no gross or histopathological signs of disease, yet significant depression of disease resistance is present and disease outbreaks become evident only when the fish are subjected to the slightest stress.

Increased levels of certain macro and micronutrients may be beneficial before and during the exposure to certain disease agents, but may be detrimental in other instances. Nutritional enhancement of disease resistance is a new field of research yet despite the need for more studies; the potential for dietary enhancement of disease resistance certainly exists.

Improving Genetic Resistance to Disease

Disease resistance can also be achieved by genetic improvement of cultured stocks. Research to improve genetic resistance to disease in farm animals has been in progress for some years now. Loses due to diseases in farm animal production are estimated at 10 to 20% of total production values. In aquaculture this figure could reach 100% during disease outbreaks. Thus breeding programs that improve disease resistance may significantly increase production values.

Selective breeding for disease resistance in fish and shellfish was given emphasis only recently. Most of the work done in fish were at first focused on improved growth rates, control of maturation, maintenance of genetic vigor and other characteristics like color (e.g. Red Tilapia), later studies then included selection for disease resistance. There are three main strategies that can be used for the improvement of disease resistance in farm and aquatic animals, namely: conventional selective breeding programs based on morphological traits; marker-assisted selection utilizing associated DNA polymorphism; and transgenic approaches.

Genetic selection for pathogen resistance in cultured shrimp species is given more attention by shrimp farmers because shrimp, unlike fish, cannot be efficiently vaccinated due to their lack of a lymphoid system that produces antibodies to the antigens of disease agents. The international shrimp breeding program has come up with a list of quantifiable immune traits for individual selection, namely: hemograms (or blood profile), hemocyte respiratory burst (related to killing ability of hemocytes), plasma antibacterial activity, levels of plasma coagulogen, and immune index (total of these quantifiable immune traits). These indexes can easily be determined by getting a sample of blood from the shrimps. Individuals scoring high in many or all these quantifiable traits can then be selected as the breeders in a breeding program.

Vaccination

Cells with the same morphology as the lymphocytes in warm-blooded animals are also present in the spleen, thymus, kidney, and blood of fishes. It is also possible to distinguish between B and T cells in fish as they are identified in higher vertebrates. Vaccination in fish is therefore possible and specific vaccines can be developed along the same principles as for warm-blooded animals. It appears that the thymus organ in young fish and the head kidney are the primary organs of lymphocyte differentiation in fish, whereas the spleen is a secondary lymphoid organ that harbors both B and T cells. The hemocytes of crustaceans do not include any cell types with properties comparable to B and T cells. Although “vaccines” for shrimps in aquaculture are being developed and marketed, such products do not fall within the current definition of a vaccine. If they reduce disease, it may be the result of a non-specific stimulation of the hemocytes by the cell wall fragments from the bacteria used in the “vaccine,” or by the adjuvants it contains and not due to a specific antibody development against the disease,

Reports on the capability of fish to produce antibodies against bacterial pathogens first came out in 1935. Then in 1942, it was demonstrated that this anti-

body response in fish translated into a protective immune response. What followed, however, was a long period of disinterest in vaccines due to the fascination of the new antimicrobial compounds (antibiotics) that came on the market immediately after the Second World War. It was only in the mid to late 1970s that attention was again given to vaccination as a means of preventing and controlling fish disease and the development of commercially available vaccines. The reasons for these turn of events were varied: the high cost of using chemotherapy, the short-term nature of the protection obtained with antibiotics, the increasing incidence of antibiotic resistant fish pathogens, and environmental concerns on the use of antibiotics.

1. Vaccine Development

Bacterial Vaccines. A number of vaccines are currently being developed against bacterial fish pathogens. Most of these works are focused on salmonid pathogens and a few are being directed against bacterial pathogens in carp and catfish. Japanese researchers are focusing their work on their cultured fish especially the yellowtail for the development of vaccines against streptococcal and *Pasteurella* infections. To date, there are still no commercially available vaccines for bacterial diseases in warm water fish.

Viral Vaccines. For some years, the only commercially available vaccine against a fish virus was the spring viraemia of carp (SVC), caused by *Rhabdovirus carpio*. It was administered by injection since the disease affects carp at a size and age (9-12 months) when they are easy to handle. However, the same is not applicable for the other important fish viruses such as infectious pancreatic necrosis virus (IPN), viral hemorrhagic septicemia (VHS) virus, infectious hematopoetic necrosis (IHN) virus, and channel catfish (CC) virus. These cause severe mortalities in fish during the fry stage in which injection is not practical. Immersion in a suspension of inactivated virus has given unsatisfactory results. Another approach had been tried using live attenuated virus or avirulent forms of the virus. Although reasonable protection has been achieved using this approach, it has been abandoned due to concerns on residual virulence in target species, virulence in non-target species, and persistence in the treated fish leading to the fear that the virus might back-mutate to virulence.

Parasite Vaccines. Parasitologists have only recently exploited the immune system to protect fish against parasitic disease. Vaccination techniques are being developed against parasitic protozoans by intraperitoneal injection of live attenuated parasites. Some evidence shows the passive transfer of protective immunity against these parasites from immune to naïve fish, and to egg. Studies are also in progress in the development of vaccines against helminthic and copepod parasites.

2. Types of Vaccines

Inactivated. The pathogenic organism is cultured *in vitro* usually in broth then killed using heat or formalin.

Live Attenuated. When the approach of using inactivated pathogens fails to elicit an immune reaction then live attenuated pathogens are used. Removing some genes from the pathogen, which is usually, a virus, making it

non-virulent develops attenuated strains. There is always the danger of back-mutation to the virulent wild type.

Recombinant. The antigens in pathogenic organisms are just minor portions of their structures, such as cell wall proteins or part of the protein coat of viruses. The genes that code for these antigenic structures can be isolated and inserted into yeast or bacterial DNA (e.g. *E. coli*) where they become incorporated and expressed in large amount. The products, usually proteins, are then harvested from the broth of cultured recombinant yeast or bacteria and used as vaccines.

DNA. The most recent approach to vaccine design is by genetic immunization or the injection of naked DNA of a pathogen into the muscle of the host. The DNA, which usually encodes a single gene of the pathogen, is expressed extrachromosomaly in the muscle cells. The newly synthesized antigen can then stimulate the immune defense of the host conferring the host with life long immunity.

3. Modes of Vaccination Delivery

The route vaccine administration depend largely on the species of fish, the size, the husbandry, the disease, the stage of the life cycle of the fish.

Direct Immersion. Vaccination by immersion is commonly practiced with very small fish where it is a convenient and highly cost-effective method of vaccine administration. Antigen uptake takes place mostly through the gills, although some may be taken up through the skin, and the lateral line and some are swallowed.

Spray Administration. Spraying a solution of a vaccine is a variation of the immersion method suitable for larger fish than those given immersion treatment. Fish are run through a conveyer belt under two or more jets containing the vaccine at 1:10 dilution for not less than 10 seconds. Antigen, like the immersion technique, is taken up through the gills.

Peroral Administration. The oral route of administration of vaccine as feed additive should have many clear advantages like the absence of handling stress, no scars on the fish to lower its value, the freedom to choose vaccination dates, no safety risk to the operator, and no risk of spreading infection through needles. However, to date, there are no cost-effective vaccines that can be administered orally.

Injection. This is the route of administration commonly used throughout the salmon industry. A single injection can provide a high degree of protection through the whole length of the culture period or production cycle. The added advantage of this method is that the process allows fish to be graded, counted, and monitored for abnormalities and signs of disease.

Immunostimulation

Immunostimulants are chemical compounds that activate the immune system of animals and increase their resistance to infectious diseases. It has been known for many years that cell wall fragments when introduced into animals will render them more resistant to pathogenic diseases. The ability of the im-

mune system to respond to microbial surface components is the result of an evolutionary process whereby animals have developed mechanisms to detect common and highly conserved chemical components of pathogenic organisms and to use these chemical components as "alarm signals" to switch on the defense mechanisms against infection. The immune system will therefore respond to an immunostimulant as if challenged by a pathogenic organism.

Immunostimulants offer many advantages when used in fish farming: 1) they may be used alone, inducing elevated activities of the non-specific defense mechanisms; 2) they promote a more effective immune response to pathogens; 3) they enhance the level and duration of the specific immune response, both cell-mediated and humoral, following vaccination; 4) they overcome the immunosuppressive effects of stress and of those pathogens that damage or interfere with the cells of the immune system.

Immunostimulants may be used prior to situations known to result in stress (handling and transfer, crowding, poor water quality, etc.) or during development stages when the animals are more susceptible to infectious diseases (larval phases, maturation and spawning, etc.). Larval or very young fish and crustaceans at all stages of their life cycle do not posses the specific (or adaptive) immune system and largely rely on nonspecific cellular defense functions to resist infections. The use of immunostimulants could improve growth and survival of juvenile fish and crustaceans. Another field where immunostimulants might be of use is in the application of antibiotics to combat infectious diseases. Most antibiotics have been proven to be immunosuppressants. A combined administration of immunostimulants and antibiotics may counteract this suppressive effect.

Substances with Immunostimulatory Effect

1. Bacterial products - these are usually cell wall components of bacteria (e.g. glycoproteins and lipopolysaccharides)
2. Products from mycelial fungi - the immunostimulants derived from mycelial fungi are all glucose polymers (e.g. lentinan, schizophyllan and scleroglucan)
3. Yeast cell wall products - these are structural components of yeast cell wall (e.g. zymosan, ß-1, 3-glucans)
4. Soluble and particle bound ß-glucans - these are animated ß-1, 3-glucans or soluble ß-1, 3-glucans bound to microbeads
5. Glycans - polysaccharides also containing sugars other than glucose
6. Chitosan – extracted from the exoskeleton of shrimps and other crustaceans
7. Peptides from animal extracts - examples of these are peptones and protein concentrates from fish, peptides extracted from the thymus of animals, the compound EF-203 from chicken eggs
8. Unspecified extracts - examples under this category are extracts from a tunicate, a peptidoglucan extract from *Bifidobacterium thermophilum*, extracts from the seeds of "malunggay" (*Moringa oleifera)*

9. Synthetic compounds - these are usually dipeptides or lipopeptides extracted from microorganisms as well as other chemical compounds that are incidentally found to have immunostimulating properties (e.g. the antihelminthic drug levamisole)

10. Cytokines - these are the molecules involved in the transmission of signals between leukocytes, such as interleukins, interferons, tumor necrosis factors, colony-stimulating factor and monocyte chemotactic factor and have dominated research in immunotherapy in humans

Biological Modification of the Culture System

Biological Filtration

Biological filters facilitate the purification of the water in high density, semi-closed or closed aquaculture facilities by the oxidation of ammonia to nitrite and nitrite to nitrate. Ammonia oxidation is accomplished by *Nitrosomonas* while nitrite oxidation to nitrate is completed by *Nitrobacter.* Both nitrite and ammonia are highly toxic compounds. The well being of the animals in the culture system depends on the ability of the biofilter to rapidly convert ammonia to nitrate which is relatively less toxic.

Ammonia is a ubiquitous by-product in an aquatic environment. It is the main excretory product of water-breathing animals. It is also the end product of the decay of organic matter. In newly established closed system the accumulation of dangerous levels of ammonia and nitrite might occur since it takes time for the biofilter to become completely colonized by *Nitrosomonas* and *Nitrobacter*. Convenient sources of nitrifying bacteria are rich garden soil (for freshwater facilities) and gravel from existing well-established biofilters. Tap water or unfiltered seawater contains these bacteria but only in small numbers. Commercial bacterial inoculates for the biofilter are also available.

Probiotics

Historically, probiotics are a group of food and feed products for both human and animal consumption and are also known as direct fed microbials. A Russian scientist who attributed the longevity of a group of Bulgarians to their consumption of fermented milk products (yogurt) first wrote the concept of probiotics about in 1908. In 1960, an Oregon microbiology professor first used the term “probiotic,” meaning for life as opposed to “antibiotic,” or against life.

In aquaculture, commercially available probiotic products contain bacterial inocula not for consumption of the fish but for the environment. Species of *Bacillus* are most commonly used, but species of *Nitrobacter, Pseudomonas, Enterobacter, Cellulomonas, Rhodopseudomonas*, and photosynthetic sulfur bacteria or their combination has been used as inocula. Some probiotic products contain enzymes or plant extracts without live bacteria.

Manufacturers of probiotics for aquaculture claim that the mode of action of their products is to enhance natural processes such as organic matter degradation, nitrification, ammonia removal, denitrification, sulfide oxidation, and degradation of toxic pollutants. They further claim that increasing the abun-

dance of useful bacteria, competitive exclusion of undesirable species, including pathogenic ones, occurs.

Polyculture

The practice of keeping several of different species in the same pond is polyculture. One of the objectives of using polyculture is to better utilize available foods in the pond. Another objective would be to control unwanted competing offspring of a cultured fish by providing a predator species. Polyculturing fish and prawns with filtering organisms to reduce phytoplankton, bacteria, and organic particles had been done on the experimental scale. Oyster, clams, macroalgae, and bloodworms are often utilized to accomplish this clean up. It is also suggested that a pond or tank containing a more varied population of fish or shellfish will also harbor a more diverse bacterial flora. This diversity may prevent the dominance of any bacterial species specially the opportunistic facultative pathogens.

Phage Therapy

Many antibacterial agents have through time aided the human race in fighting bacterial diseases. In the last part of the 1800's and in the early 1900's scientists have discovered viral particles that had a killing effect on bacteria. These particles were named bacteriophage, or phage, for their ability to "eat" bacteria. Phage therapy is the use of bacteriophage to treat bacterial disease. The development of antibiotics in the 1940's directed attention away from bacteriophages as a mode of treatment. However, the recent concern regarding bacterial resistance to antibiotics has prompted a renewed interest in bacteriophage as biological control of bacterial pathogens.

Each kind of bacteria has its own phages, which can be isolated wherever that particular bacterium grows - from sewage, feces, soil, the sea, ocean depths and hot springs. Phages are specific to their bacterial host because of a particular protein antibody on the surface of the bacteria that a phage will specifically bind to. Since phages are specific they cause much less damage to the normal microbial balance of the host. Moreover, phages replicate within the body of the infected animal thus requiring only a single dose of phage that multiply only as long as the target bacteria are present.

Most of the work on phage therapy was conducted in Eastern Europe and all these concentrated on common human bacterial disease. Although phage therapy is still not practiced in agriculture and aquaculture, it shows great potential as a safe, specific and cheap alternative to chemotherapeutics.

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CHAPTER TEN

Immunological and molecular biology techniques in disease diagnosis

Leobert D. de la Peña

Traditionally, the diagnosis of infectious diseases has been accomplished by the isolation of the infecting microorganism in pure culture. Classical methods of microbial isolation and identification have been invaluable in the study of bacterial, viral and fungal infections. However, cultivation systems offer disadvantages for the rapid diagnosis of infectious diseases. For example, many microorganisms, especially viruses and slow growing bacteria, require a considerable period of time in cultivation that the results from cultures are often not available at a time when the result can alter the course of therapy. Thus, more sensitive means must be applied for detecting and identifying a wide range of infectious diseases of fish and shrimps.

The development of rapid, simple, sensitive and specific diagnostic tests for infectious diseases has been much slower than the other disciplines of medicine. This is partially attributed to the complexity and diversity of the pathogenic organisms and to the difficulty in detecting low concentrations of these organisms in samples. Immunological and molecular biology-based techniques are rapidly advancing the field of diagnostics in fish and shrimp diseases.

IMMUNOLOGICAL TECHNIQUES

Antigen-antibody reactions are most easily studied *in vitro* using preparations of antigens and antisera. Reactions of antigens and antibodies are highly specific. An antigen will react only with antibodies elicited by itself or by a closely related antigen. Because of the high specificity, reactions between antigens and antibodies are suitable for identifying one by using the other. However, cross-reactions between related antigens can occur, and these can limit the usefulness of the test. The study of antigen-antibody reactions *in vitro* is called serology, and is important in clinical diagnostic microbiology.

Immunodiagnostic tests use an antigen-antibody reaction to detect and identify a specific antigen or antibody associated with a disease-causing organism. The antigen-antibody reaction itself is very specific. Consequently, if the correct antibodies can be obtained, immunodiagnostic techniques have the advantage of being able to identify the presence of a specific pathogen directly in the

specimens and also can be used to detect the specific antibodies produced as a result of the immune response of the host to the organism. The primary component in the test is the antibody. The specificity, and to some extent the sensitivity, of the assay depends on the quality of the antibodies used in the reagents.

Antibodies are formed by clonal selection as explained in Chapter 9. A large pool of B lymphocytes (B cells) display immunoglobulin (Ig) molecules on their surface. These Ig serve as receptors for a specific antigen, so that each B cell can respond to only one antigen or a closely related group of antigens. An antigen interacts with the B lymphocyte that shows the best "fit" by virtue of its Ig surface receptor. The antigen binds to this receptor, and the B cell is stimulated to divide and form a clone. Such selected B cells soon become plasma cells and secrete antibody. Since each person can make 10^7-10^8 different antibody molecules, there is an antigen-binding site on a B cell to fit almost any antigenic determinant.

Antibodies are immunoglobulins that reacts specifically with the antigen that stimulated their production. Antibodies that arise in an animal in response to a single antigen are heterogeneous because they are formed by several different clones of cells; i.e., they are polyclonal antibodies. Antibodies that arise from a single clone of cells, e.g., in a plasma cell tumor (myeloma), are homogeneous; i.e., they are monoclonal antibodies. Monoclonal antibodies can be made by fusing a myeloma cell with an antibody-producing lymphocyte. Such hybridomas produce virtually unlimited quantities of monoclonal antibodies *in vitro*. Monoclonal antibodies excel in the identification of antigens because cross-reacting antibodies are absent thus they are highly specific.

There are two basic methods for identification of unknown organisms by using sera containing various known antibodies; one is agglutination for particulate antigens and another is precipitation for soluble antigens. Other immunoassay methods include, fluorescent antibody technique (FAT), enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

Agglutination

Agglutination reactions are among the most easily performed of immunological tests. Their usefulness in presumptive identification of bacteria has long been recognized and for bacterial fish pathogens has been reconfirmed and documented. With as few as a dozen antisera, it is possible to confirm the identity of the majority of known bacterial fish pathogens. Agglutination has provided valuable information on the serological relation of bacterial fish pathogens, including species within genera and strains of the same species.

In this reaction, the antigen is particulate (e.g., bacteria and red blood cells) or is an inert particle (latex beads) coated with an antigen. Because it is divalent or multivalent, the antibody cross-links the antigenically multivalent particles and forms a latticework, and clumping (agglutination) can be seen (Figure 10-1). The procedure is used both to demonstrate the presence of antibodies in serum and to identify antigens on microbial cell surfaces. The principle is the same in both applications. If the serum contains antibodies against a surface antigen, they will agglutinate the bacterial cells. Using a variety of typing sera,

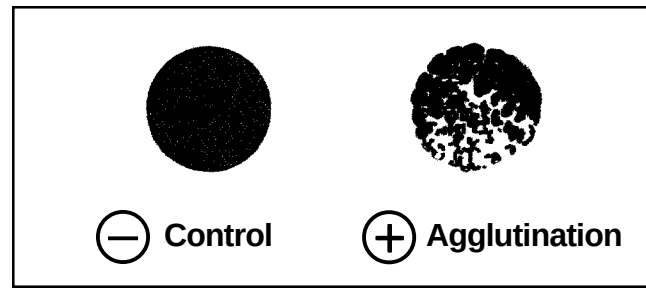

Figure 10-1. Slide agglutination. Negative (-) and positive (+) reactions

bacteria can be identified and classified. If a constant amount of a bacterial cell suspension is mixed with graded volumes of dilutions of homologous antiserum, one obtains a measurement of the concentration of antibodies in the serum. The term used to describe the concentration of antibody in serum is "titer", which is the reciprocal of the highest dilution producing a definite reaction.

For use in agglutination tests, bacteria harvested from broth or agar medium may be resuspended adequately. Sometimes bacteria have a tendency to aggregate spontaneously, or "autoagglutinate", making their use in agglutination tests unfeasible. If spontaneous agglutination is observed with a bacterial suspension to be used as a test organism, there are several modifications that can be used to eliminate this reaction. If the preparation was formalinized before removal from the growth medium, sometimes washing the bacteria in neutral buffered saline before formalinization reduces non-specific agglutination. If agglutination still occurs, resuspension of the organisms in a 0.1% protein solution such as non-fat dry milk to block sites of adherence may be a remedy. Further autoagglutination may necessitate heating of bacterial suspension by immersion in a boiling water bath for 1 to 10 minutes. If none of these procedures eliminate the autoagglutination problem, the particular organism may not be amenable to this procedure.

Agglutination test has been widely used in detecting bacterial fish pathogens belonging to the genera *Vibrio*, *Pasteurella*, *Aeromonas*, *Yersinia*, *Edwardsiella* and *Pseudomonas* (Toranzo et al., 1987).

Precipitation

In this reaction, the . The antibody cross-links antigen molecules in variable proportions, and aggregates (precipitates) form. In the zone of equivalence, optimal proportions of antigen and antibody combine; the maximal precipitates forms, and the supernatant contains neither an excess of antibody nor an excess of antigen. In the zone of antibody excess, there is too much antibody for efficient lattice formation, and precipitation is less than maximal. In the zone of antigen excess, all antibody has combined but precipitation is reduced because many antigen-antibody complexes are too small to precipitate.

Procedures involving soluble antigens have been used to study the antigenic composition of fish pathogens (bacterial, viral and parasite) and to a lesser extent a diagnostic tools.

Precipitin reactions can be done in semisolid medium (agar):

1. Single diffusion

Using this method, it is possible to quantitate the concentration of anti body and antigen in a solution. The antigen diffuses in a radial direction out from the well and a precipitin ring develops when the reactants are close to their optimal proportions (Figure 10-2). At the equivalence point, when the ring is stationary, the square of the diameter or area of the ring is directly proportional to antigen concentration. Conversely, though less sensitive, antigen can be mixed into the agar and the amount of antibody in a sample can be determined. Consequently, it is possible to calibrate the plate using a pre-determined constant amount of antibody (or antigen) in the agar and placing known concentrations of antigen (or antibody), or sample dilutions in the wells. By calibrating the method, such radial immunodiffusion is used to measure IgG, IgM, complement components, and other substances in the serum.

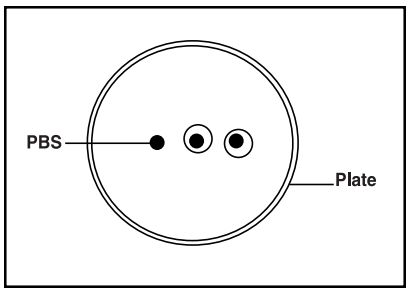

Figure 10-2. A representation of a single radial immunodiffusion measurement. Typical rings obtained with positive rabbit serum and no ring with phosphate buffered saline (PBS) as negative control

In some cases, poor resolution occurs with complex antigenic mixtures although the method is relatively sensitive when soluble antigens are employed. Furthermore, the length of time for visualization of results depends on antigenic type and/or molecular mass. This technique is best used with low molecular weight antigens and is unaffected by molecular charge. With high molecular weight antigens, the diffusion time is longer for formation of the precipitin ring maximum diameter. The use of tannic acid as a precipitin intensifying agent increases the plate sensitivity and allows detection of lower antigen levels in serum. The method offers a limited benefit and is insensitive for diagnostic purposes. It requires higher antigen concentrations in the test mixture since low amounts may result in lack of precipitation of the samples used.

The method has been used infrequently in fish immunological studies and then only for estimation of the immunoglobulin concentrations in both normal and immune serum. The concentration of immunoglobulins produced in response to injection with *Salmonella* bacterial and/or red blood cell (RBC) antigens have been measured in catfish (Ourth, 1986). Serum IgM levels have been measured in rainbow trout naturally infected with VHS virus and ERM bacteria (Olesen and Jorgensen, 1986).

2. Double diffusion

In the gel diffusion technique, gels, usually clarified agar are used as matrices for combining diffusion with precipitation. Antigen and antibody are placed in different wells in agar and allowed to diffuse towards each other (passive diffusion) and precipitation results where the optimal antibody/antigen ratios have been reached (Figure 10-3). This method (Ouchterlony) can be used either to detect the number of major components in an antigenic mixture or identify the presence of homologous and heterologous molecules in an antigenic extract based on the specific recognition capacity of a prepared antiserum.

If two different samples contain identical or related antigen epitopes, the precipitin lines are confluent and meet to give a single arc, thus a denoting a

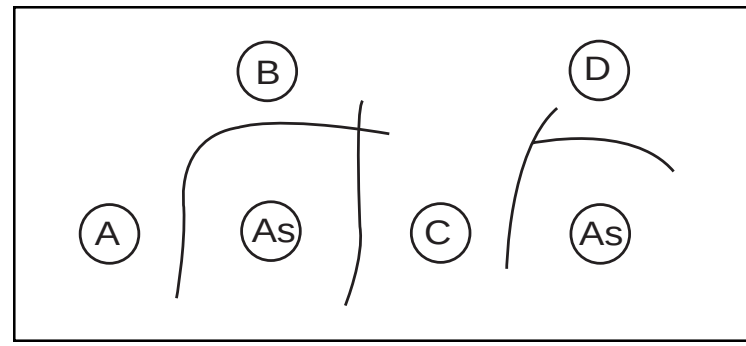

Figure 10-3. Double diffusion precipitation reactions (Ouchterlony) in gel. As – antiserum in wells; A, B, C, D – antigens in wells; A and B – reaction of identity; B and C reaction of nonidentity; C and D – reaction of partial identity (cross-reaction; the "spur" is caused by the fraction of antibody that was not precipitated by antigen D)

reaction of identity. If antigens in the two samples are dissimilar or unrelated and possess no common epitopes then the precipitin lines are independent of each other and cross over, indicating a reaction of non-identity. When the antigen samples contain two molecular species, a reaction of identity occurs when epitopes are similar to both of the molecular types but an additional epitope on one of the antigen species would give rise to a line of non-identity such that a spur is formed. This is also referred to as a reaction of partial identity since the antisera would detect the unique component on one but not the other antigen of similarity. Lastly, if the two molecular species have one epitope in common and each has an epitope(s) not present in the other, partial identity occurs and two spurs are formed.

The method has been employed frequently to determine the serological relationships between fish bacterial strains on the basis of lipopolysaccharide types, between *Vibrio* bacteria isolated from other marine teleosts (Johnsen, 1977), in the identification of extracellular *Vibrio* toxins pathogenic to eels and ayu (Kanemori et al., 1987) and for comparison of antigenicity of *Edwardsiella tarda* after injection into ells (Salati and Kusuda, 1985). It was also used to diagnose BKD (Kimura et al., 1978) and to determine the serological differences of *Photobacterium damsela* subsp. *piscicida* isolates (Kawahara et al., 1998).

3. Immunoelectrophoresis

This technique is a widely used method in fish immunology and combines electrophoresis with immunoprecipitation. It has a much better resolution than gel diffusion. In this system, the components of the antigen will first be separated by electrophoresis. Separation of the components occurs due

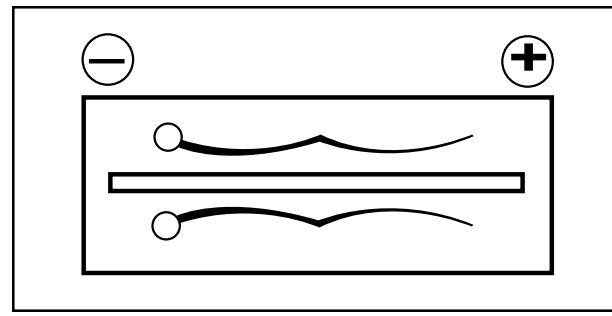

Figure 10.4. Immunoelectrophoretic pattern of *Vibrio harveyi* LPS. In the well above the trough, LPS in the supernatant after ultracentrifugation of the water layer while in the well below the trough, purified LPS. In the trough, anti-*V. harveyi* serum

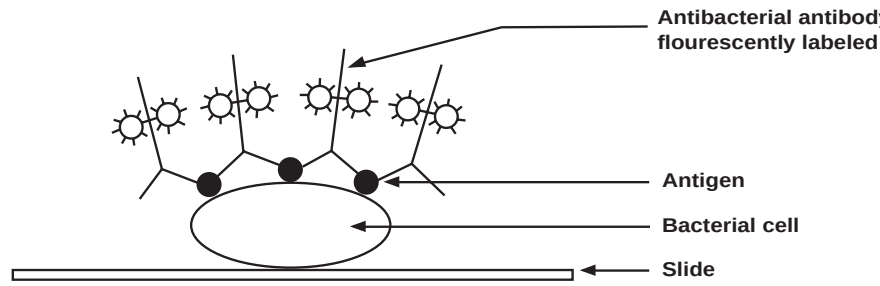

Figure 10-5. Direct fluorescent antibody technique (DFAT)

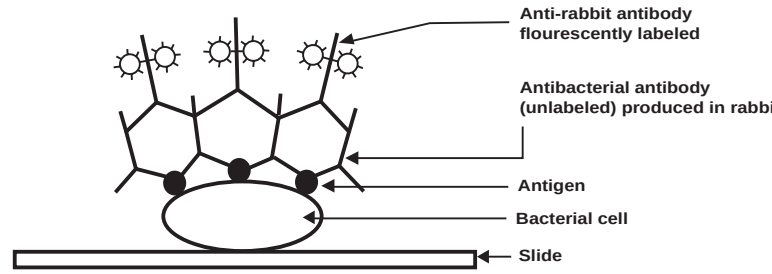

Figure 10-6. Indirect fluorescent antibody technique (IFAT)

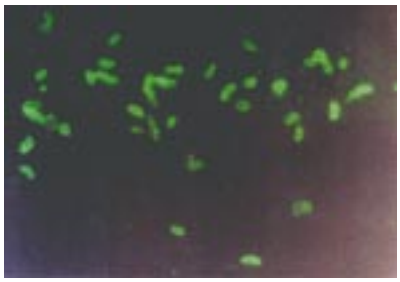

Figure 10-7. Indirect fluorescent antibody technique (IFAT) staining of an impression smear prepared from *Vibrio penaeicida* cell suspension

to different electrophoretic mobilities caused by charges on the molecules. After the antigen has been separated into its components, antiserum will be put into a channel cut parallel to the direction of the electrophoresis. From this channel, the antibodies will diffuse torwards the electrophoretically separated antigen components, and vice versa. As the antigen and antibody diffuse toward each other, they form a series of arcs of precipitate (Figure 10-4). This permits the serum proteins to be characterized in terms of their presence, absence, or unusual pattern. The separated components are then visualized on the plate by precipitin band formation due to the diffusion of a specific antiserum into the agar parallel to the current direction. With this method, the antigenic complexities of various sera can be determined, detect the purity of one component system or isolated fractions, detect and to determine the number of components in a multicomponent system and to demonstrate antibody heterogeneity.

The test was used to identify IPN virus in cell culture (Dea and Elazhary, 1983) and determine the serological characteristics of atypical strains of *Edwardsiella tarda* isolated from sea breams (Costa et al., 1998).

Fluorescent Antibody Technique (FAT)

Searching for bacteria in fish tissue is easier than defining viral infections by FAT. In most cases when antisera against viral fish pathogens are used, the fish tissues suspected of being infected are processed, filtered and placed in culture on fish cell lines; the purpose being to increase the amount of virus.

The FAT makes use of the feature that some dyes (fluorescein isothiocyanate, rhodamine isothiocyanate, Texas red) fluoresce when viewed under ultraviolet

(UV) light. These dyes can be used as tags, conjugated to target antibody molecules, and when the antibody complexes with the specific antigen, the complex is "lighted". A major advantage of being able to visualize the antigen-antibody reaction on the pathogen gives the pathologist confidence in diagnosis. This technique generally detects surface-associated antigens. Slide formats, with organisms fixed directly to the slides, are used for bacteria. After fixation, antigen is incubated with specific antibody. This may be labeled directly with fluorescein to allow visualization of antibody (direct FAT) (Figure 10-5) or, alternatively, a fluorescein-labeled antibody conjugate is added in a second stage to detect bound antibody (indirect FAT) (Figures 10-6 and 10-7). Slides are then examined with an ultraviolet microscope, and the intensity of fluorescence, relative to appropriate controls, is scored in an arbitrary scale from -, +, to + + + + +. Typical assays can be performed in 2-3 h.

Immunofluorescence is widely used for the detection of antigen or antibody in fish, typing and identification of a range of microorganisms. FAT has been used to detect antibodies to *Aeromonas liquifaciens* in fish (Lewis and Savage, 1972). Rapid FAT diagnosis has been developed to detect Pseudotuberculosis in yellowtail (Kitao and Kimura, 1974), *Renibacterium salmoninarum* in salmonids (Bullock et al., 1980) and *Vibrio penaeicida* in kuruma prawn (de la Peña et al., 1992). FAT has also been developed for rapid diagnosis of infectious hematopoeitic necrosis virus (IHNV) (LaPatra et al., 1989) and red sea bream iridovirus (Nakajima et al., 1995) in fish.

Enzyme-Linked Immunosorbent Assay (ELISA)

The covalent attachment of enzymes to antibody molecules creates an immunological tool possessing both high specificity and sensitivity. The technique makes use of antibodies to which enzymes have been covalently bound such that the enzyme's catalytic properties and the antibody's specificity are unaltered. Typical linked enzymes include peroxidase, alkaline phosphatase and b-galactosidase, all of which catalyze reactions whose products are colored and can be measured in very low amounts.

The ELISA is one of the most powerful of all immunochemical techniques. It employs a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens. There are many variations on the ways the immunoassays can be performed. Immunoassays are classified on

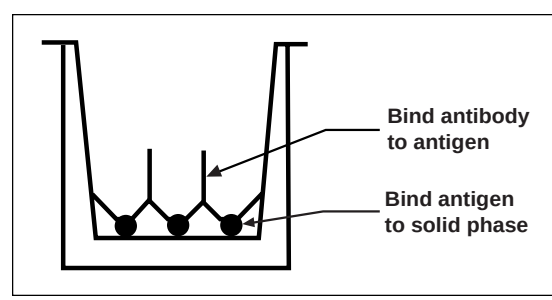

Figure 10-8. Antibody-capture assay

the basis of methodology and within each group, the principle and the order of the steps are similar. For example, by changing certain key conditions, an assay can be altered to determine either antigen or antibody level. Although the steps are similar, the assays yield different results.

The three classes of ELISA tests are:

1) antibody capture assays,
2) antigen capture assays and
3) two-antibody sandwich assays

Antibody capture assay can be used to detect and quantitate antigens or antibodies and compare the epitopes recognized by different antibodies. The general protocol; an unlabeled antigen is immobilized on a solid phase and the antibody is allowed to bind to the immobilized antigen (Figure 10-8). The antibody can be labeled directly or can be detected by using a labeled secondary reagent like goat anti-rabbit or anti-mouse IgG antibody containing conjugated enzyme that will specifically recognize the antibody. Following the addition of enzyme substrate, a color is formed, and the amount of antibody relative to the specific antigen is quantitated from the intensity of the color reaction measured by an ELISA plate reader, a modified spectrophotometer The color formed is proportional to the amount of antibody that is bound. The three factors that will affect the sensitivity of a labeled antibody assay are (1) the amount of antigen that is bound to the solid phase, (2) the avidity of the antibody for the antigen and (3) the type and number of labeled moieties used to label the antibody. Variations in methodology under antibody capture assay are (1) detecting and quantitating antibodies using antigen excess assays, (2) comparing antibody binding sites using an antibody competition assay, (3) detecting and quantitating antigens using antibody excess assays and (4) detecting and quantitating antigens using antigen competition assays.

Antigen capture assays are used primarily to detect and quantitate antigens (Figure 10-9). The amount of antigen in the test solution is determined using a competition between labeled and unlabeled antigen. Unlabeled antibody is bound to the solid phase either directly or through an intermediate protein, such as an anti-immunoglobulin antibody. The antigen is purified and labeled. A sample of the labeled antigen is mixed with the test solution containing an unknown amount of antigen and the mixture is added to the bound antibody.

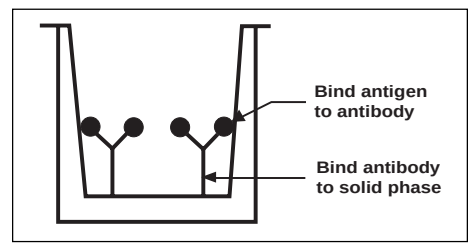

Figure 10-9. Antigen-capture assay

The antigen in the test solution will compete with the labeled antigen for binding to the antibody-matrix. Unbound proteins are removed by washing, and the amount of labeled antigen bound to the matrix is measured. If the un-

known solution contains a high concentration of antigen, it will compete effectively with the labeled antigen and little or none of the labeled antigen will bind to the antibody. Following a wash, the enzyme activity of the bound material in each microtiter well is determined by adding the substrate of the enzyme. Color development in individual wells of the plate is assessed by eye or more commonly, quantified with a commercially available ELISA plate reader-a modified spectrophotometer The color formed is proportional to the amount of antigen originally present. It is necessary to determine cut-off values to discriminate between true positive reactions and background reactivity by including appropriate positive and negative controls with each series of assays. The sensitivity of the labeled antigen assay will depend on three factors: (1) the number of antibodies that are bound to the solid phase, (2) the avidity of the antibody for the antigen and (3) the specific activity of the labeled antigen.

Two-antibody sandwich assays are used primarily to determine the antigen concentration in unknown samples. The assay requires two antibodies that bind to non-overlapping epitopes on the antigen (Figure 10-10). Either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies can be used. To use the assay, one antibody is

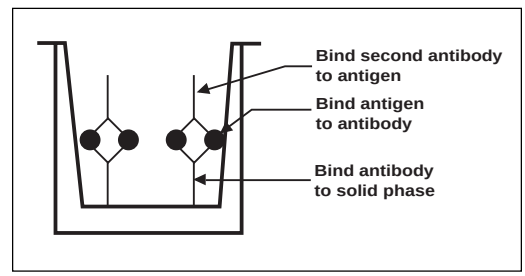

Figure 10-10. Two-antibody sandwich assay

purified and bound to a solid phase and the antigen in a test solution is allowed to bind. Unbound proteins are removed by washing and the labeled second antibody is allowed to bind to the antigen. The conjugate comprises antibody chemically linked to the enzyme horseradish peroxidase. After a further incubation, excess conjugate is removed by washing the wells and the bound peroxidase is determined by adding chromogen in substrate buffer. Color development (blue) is proportional to the original bacterial content in the sample and can easily be read by the naked eye. The assay can also be amplified at this stage thus increasing sensitivity by adding stop solution. The color obtained changes from blue to yellow and results are read using an ELISA plate reader with a 450 nm filter. The inclusion of appropriate positive and negative controls are essential. In addition, during the optimization of the test, check for endogenous peroxidase activity in the tissue samples being used. If activity is found, then this may be overcome by altering the method of extraction (e.g. heating step). It may be necessary to change to a different enzyme system (e.g. alkaline phosphatase) if activity is very high (e.g. spleen tissue). The major advantages of this method are that the antigen does not need to be purified prior to use and that the assays are very specific. The major disadvantage is that not all antibodies can be used. The sensitivity of the assay is dependent on four factors: (1) the number of molecules of the first antibody that are

bound to the solid phase, (2) the avidity of the first antibody for the antigen, (3) the avidity of the second antibody for the antigen and (4) the specific activity of the labeled second antibody.

Two types of detection systems are commonly used for ELISA: iodinated reagents and enzyme-labeled reagents. Assays that use iodinated reagents are easier to quantitate while enzyme assays will often yield a quicker result. Either iodinated or enzyme-labeled reagents can be used for direct or indirect methods. When using direct detection methods, the antibody or antigen is purified and labeled while for indirect detection, a labeled secondary reagent that will bind specifically to an antibody is used. A third variation that uses properties of both direct and indirect detection is the biotin-streptavidin system. Here, the antigen or antibody is purified and labeled with biotin. The biotinylated reagent is detected by binding with streptavidin that has been labeled with iodine or an enzyme.

ELISA test has been used to detect *Aeromonas salmonicida* in fish tissue (Adams, 1990), clinical cases of enteric red mouth and furunculosis in fish farms (Austin et al., 1986), *Vibrio parahaemolyticus* (Adams, 1991) and *V. harveyi* (Song et al., 1992) in penaeid shrimp. ELISA has also been developed for rapid detection of viral haemorrhagic septicaemia virus (Olesen and Jorgensen, 1991) and striped jack nervous necrosis virus (Arimoto et al., 1992) in fish.

Western Blotting

Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. The technique allows one to identify particular proteins by utilizing the specificity inherent in antigen-antibody recognition. This technique is powerful, since it combines electrophoretic separation of proteins, glycoproteins and lipopolysaccharides with immunological identification. Once such antigens have been detected, they can be further characterized by Western blotting. Both techniques can utilize either polyclonal or monoclonal antibodies.

Initially, a sample is subjected to electrophoresis to separate antigens according to their charge and size, or size alone. A second electrophoretic step transfers the antigens from the gel to an immobilizing surface, such as nitrocellulose

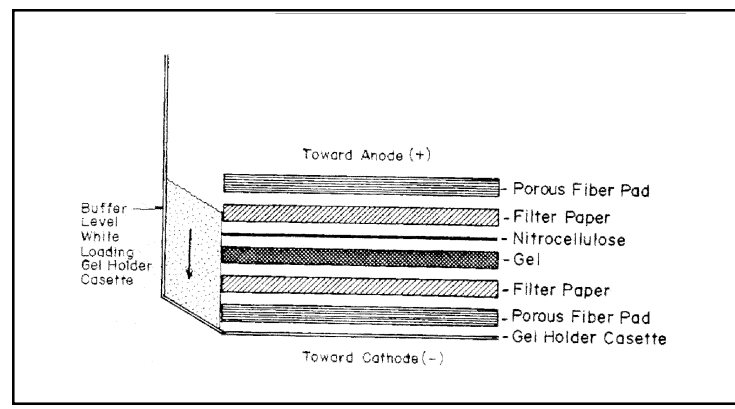

Figure 10-11. Western blotting. Organization of materials inside the gel cassette holder for transfer of proteins from the gel to nitrocellulose paper

paper where they are bound irreversibly (Figure 10-11). After this transfer, the paper is blocked with 3% gelatin in PBS to prevent nonspecific binding of antibody and probed with a specific enzyme-conjugated antibody (horseradish peroxidase-anti-immunoglobulin conjugate). A chromogenic substrate is then added to determine which electrophoretic band is bound by the antibody.

The technique is useful for a number of purposes including characterization of unknown antigens or antibody specificities, confirmation of the presence of bacterial antigens in sera or tissues and detection of seropositive individuals which have been exposed to a pathogen. The primary advantage of Western blotting, as opposed to other immunoassays, is the high degree of specificity in resolving distinct antigens. However, there are two disadvantages: first, it is mainly a qualitative assay and quantification of antibody or antigen is difficult; and second, if the antigen sample must be denatured (such as in SDS-PAGE), antigenic activity may be reduced or destroyed. An initial consideration for successful Western blotting is the empirical determination of the optimal percentage of acrylamide for resolution of the antigens of interest. Next, it must be determined whether the antigens are capable of binding to the nitrocellulose. Finally, the antigen detection procedure must be highly specific and sensitive.

The technique can be used to detect as little as 1 ng of a protein antigen that has been previously separated under denaturing conditions by SDS-PAGE, provided that an antibody that recognizes the denatured form of the protein is available. The entire procedure can be completed in 1 to 2 days, depending on transfer time and type of gel.

Western blotting has been useful for characterizing the specificities of polyclonal antisera (rabbit and salmonid) and monoclonal antibodies to extracellular and cell surface antigens of *Renibacterium salmoninarum* (Wiens and Kaattari, 1989). It has also been used in the detection of yellowhead virus and white spot syndrome virus in penaeid shrimp (Nadala et al., 1997; Magbanua et al., 2000).

MOLECULAR BIOLOGY TECHNIQUES

The central foci of molecular biology are the nucleic acids, deoxyribonucleic acid (DNA) and the ribonucleic acid (RNA). Nucleic acids encode the genetic information specifying the primary structure of all proteins unique to an organism. Together with lipids and extracellular supporting stroma, they create cellular activity and physiological function. Thus, biological functions can be understood in part by examining the interrelationships between these key components. RNA and DNA are composed of four separate building blocks called nucleotides. Each of the four nucleotides contains a nucleic acid base (A, adenine; G, guanine; T, thymine; C, cytosine), a deoxyribose sugar moiety and a phosphoester for DNA. For RNA, the same bases as in DNA are present, except that uridine (U) is substituted for T and a ribose moiety is present instead of the deoxyribose. The nucleotides are connected one to another to form a chain-like arrangement, which comprises the nucleic acid's sequence. RNA is

composed of a single strand whereas DNA is composed of two paired strands. In order for the paired strands to match up, they must face each other in the opposite or complementary direction. The complementary strands of DNA are kept together primarily by the hydrogen bonds that form between the bases A and T (2 bonds) as well as C and G (3 bonds). It is this hydrogen bonding between the matched base pairs A and T (or, for RNA, A and U) as well as C and G that is the foundation of all molecular biological tests. Although one given base pair match of AT or GC would separate easily, there is strength in numbers and the more base pair matching, the greater the number of hydrogen bonds between the two DNA strands and the less likely they are to separate.

In the hybridization of nucleic acid strands, when two DNA strands meet, they orient each other in opposite or antiparallel directions to allow base pair matching to occur. If no base pair matching is present, they go their separate ways. However, if there is sufficient base pair matching they will join together of hybridize. The specific term used to describe the degree of base pair matching that determines if the strands stay together is homology. How much homology is needed for two strands to stay together? Although it is true "the more, the better," another important variable is how close the base pair matches are to one another. Adjacent base pair matches in a sequence will hold together more strongly than the same number of base pair matches dispersed over the DNA sequence. Dispersed base pair matches are typical unrelated DNA strands whereas clustered base pair matches are expected for related complementary DNA molecules. Clearly, if two DNA strands are completely homologous and have 100% base pair matching then the strands would tend to remain hybridized under most conditions. Conversely, hybridized strands with poor homology (e.g., only 10% of base pairs matched) would tend to dissociate or denature readily under most conditions. Whether hybridized strands with intermediate homology - where, for example, 50% of the base pairs matched - would remain hybridized would depend greatly on the reaction conditions.

Given that hydrogen bonds are the glue that keep two hybridized strands together and that many chemicals and conditions can affect hydrogen bonding, a term is needed that describes whether the hybridization reaction conditions relatively favor or disfavor hydrogen bonding - stringency. Under low stringency conditions, hybridized strands with intermediate homology would tend to remain hybridized whereas hybridized strands with poor homology would dissociate. At high stringency conditions, only hybridized strands with strong homology would tend to remain hybridized.

Another key term, the melting temperature, or commonly abbreviated, T_m. If one takes two strands of DNA that share homology and hybridizes them, at any given time some of the strands will remain hybridized whereas others will have separated. The ratio of hybridized/denatured DNA strands in the reaction will vary depending on the degree of homology as well as any condition that may affect hydrogen bonding between matched base pairs such as formamide concentration and temperature. The melting temperature is defined as that temperature under the specific reaction conditions where one half of the hybridized strands are still hybridized and the other half are denatured.

Two molecular biology-based techniques discussed here are: gene probe and polymerase chain reaction (PCR).

Gene probe assays

The power of DNA diagnostics is a consequence of two facts: (1) nucleic acids can be rapidly and sensitively measured, and (2) the sequence of nucleotides in a given DNA molecule is so specific that hybridization analyses can be used for reliable clinical diagnoses.

One of the most powerful analytical tools available is nucleic acid hybridization. Instead of detecting a whole organism or its products, hybridization detects the presence or absence of specific DNA sequences associated with a specific organism. To identify a microorganism through DNA analysis, you must have available nucleic acid probe to that microorganism, a single strand of DNA containing sequences unique to the organism. The unlabeled strand in the sample being analyzed that is homologous to the probe is called the target. If a microorganism in a specimen contains DNA sequences complementary to the probe, the two sequences can hybridize forming a double stranded molecule. To detect that a reaction has occurred, the probe is labeled with a **reporter** molecule, either a radioisotope, an enzyme, or a fluorescent compound that can be measured in small amounts following hybridization. Depending on the reporter used (radioisotopes are the most sensitive), as little as 0.25 mg of DNA per sample can be detected.

Several techniques have evolved based on the ability of a labeled probe to bind to and thus permit the detection of the target nucleic acid sequence of interest. One approach is to extract the DNA, both target and non-target, from a sample and bind it to a filter where it can be hybridized with the labeled probe. This is called filter hybridization. Often times the sample DNA is directly placed on the filter with the aid of a vacuum manifold, which has slot-like spaces for each sample, hence the term slot blot or dot blot hybridization. Dot blotting is rapid, simple technique for the quantification of RNA or DNA target sequences without prior electrophoretic separation. This method differs only in the shape of the immobilized nucleic acid spot deposited on the membrane. Nucleic acid is applied to a dry nitrocellulose filter and allow to dry. The resulting “dots” are variable in size, making accurate estimates of target sequence concentration difficult. Alternatively, the sample DNA may first be separated according to size and configuration by electrophoresis on a gel and then transferred to a filter - this is termed Southern blot hybridization. As can DNA fragments, RNA molecules can be separated on the basis of size by gel electrophoresis and can be immobilized on membranes by a process referred to as Northern blot hybridization. Detection of target sequences in Southern, Northern and dot blots is carried out under essentially identical conditions. Three basic processes are involved: (1) prehybridization, which saturates nonspecific DNA binding sites on the membrane with random DNA and polymers; (2) hybridization, during which specific labeled probes are annealed to target sequences; and (3) washing, to remove unhybridized and imprecisely hybridized probe. In either of these techniques, the tissue must be destroyed thus precluding direct histological correlation.

In the other major strategy based on hybridization of a target and a probe, the target DNA is not extracted but rather kept in the intact cell where it may bind to the probe - this is the *in situ* hybridization. The principal advantage of *in situ* over other molecular techniques is it provides information about the location of the target nucleic acids within cells and/or tissues. Pathologists are able to look for specific nucleic acids and to study the cellular and tissue morphology of the sample. A misconception about *in situ* hybridization is that one needs to use radiolabeled probes in order to maximize its sensitivity however, recent and dramatic advances in nonisotopic labeling and, more importantly, detection systems has greatly enhanced the sensitivity using such common labels as biotin and digoxigenin. The most common problem encountered with the use of nonradioactive systems is background. Background may be defined as the presence of a hybridization signal with a specific probe in areas of the tissue where the signal should not be present. Background is often the result of nonspecific binding of the probe to nontarget molecules. Two ways to deal with background are to decrease the concentration of the probe and/or to increase the stringency of the post-hybridization wash. Another common problem is poor tissue morphology due to overtreatment with the protease solution. Decreasing the time of digestion or the concentration of the protease tenfold will solve this problem. Another is occasionally tissue sections may fall off. The problem rests with incorrect silanization of the slides. The most obvious potential problem is the absence of a hybridization signal. In dealing with a negative signal, you have to check the proper fixative (no heavy metals or picric acid), alkaline phosphatase conjugate, chromagen, denaturing temperature (= 95°C) and the tissue was deparaffinized. The target molecules are nucleic acids. Nucleic acids are complexed with proteins in the cell; when a tissue is embedded in a complex matrix, the nucleic acids are cross-linked to that matrix. Thus, major challenges of *in situ* hybridization are to make target nucleic acid available to the probe; and once proper hybrids are formed, to stabilized them without destroying the cell morphology.

An important difference between filter and *in situ* hybridization is the detection threshold. Detection of a DNA sequence by *in situ* hybridization implies a selective increase in its numbers due to, for example, oncogene amplification or viral proliferation. On the other hand, only one virus need to be present per every 100 cells for the Southern blot test to detect it, though the *in situ* test would be scored as negative. *In situ* is relatively insensitive test and is usually negative in situations such as occult or latent infection by a virus where the number of target DNA sequences per cell is low. Why is there such a disparity in detection threshold? The primary reason is the probe may find it more difficult to find the target if it has to traverse the labyrinth of nuclear proteins and nucleic acids in *in situ* analysis relative to more “naked” DNA that has been attached to a filter in dot blot or Southern blot hybridization. Second, the extraction and purification of DNA characteristic of filter hybridization leads to a concentrating effect of rare nucleic acid sequences.

Nucleic acid probes offer many advantages over immunological assays. Nucleic acids are much more stable than proteins to high temperatures, high pH, organic solvents and other chemicals. In addition, nucleic acid probes are more defined entities than antibodies.

Gene probes are extensively used as diagnostic tools to detect white spot syndrome virus (WSSV) (Chang et al., 1996; Chang et al., 1998), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Mari et al., 1993) and hepatopancreatic parvovirus (HPV) (Mari et al., 1995) of penaeid shrimp and viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic necrosis virus (IHNV) in fish (Ristow et al., 1991).

Selected features of three major hybridization assays

	In situ	Southern blot	Dot blot
Detection threshold	20 copies/cell	1 copy/100 cells	1 copy/200 cells
Samples	fresh or fixed	fresh	fresh
Background	low	low	low-high[a]
Time	≤ 1 day	2-7 days	1-5 days
Cell localization	yes	no	no
Equipments	none	transfer unit gel cast/electrophoresis unit	vacuum manifold
Samples/run	≤ 25	≤ 15	≤ 75
Detection of latent infection	no	yes	yes

[a]Depending on stringency.

Polymerase Chain Reaction (PCR)

Various molecular biology techniques continue becoming more important in fish and srimp farming, particularly in detection and prevention of various diseases. One of the most prominent techniques is the PCR. No technique has had a greater impact on the practice of molecular biology than the PCR. The PCR for amplification of specific nucleic acid sequences was introduced by Saiki et al. (1985) and has subsequently proved to be one of the most important scientific innovations of the past decade. With this technique, one can rapidly detect a virus or bacteria, few copies of mRNA, rapidly synthesize, clone and sequence virtually any segment of DNA. Despite the incredible power of the technique there has been one major limitation that is the DNA must be extracted from the sample one can not correlate PCR results with the pathological features of material being tested.

The development of PCR means that small amounts of DNA no longer limit molecular biology research or DNA-based diagnostic procedures. The technique is continuously improving and its full impact on molecular diagnostics is yet to come. PCR currently has many applications, including analysis of ancient DNA from fossils, amplification of small DNA amounts for analysis by DNA fingerprinting, mapping the human genome and also those of other species, and detection of microorganisms present in low densities in water, food, soil or other organisms. In aquaculture, PCR is a valuable tool for the preven-

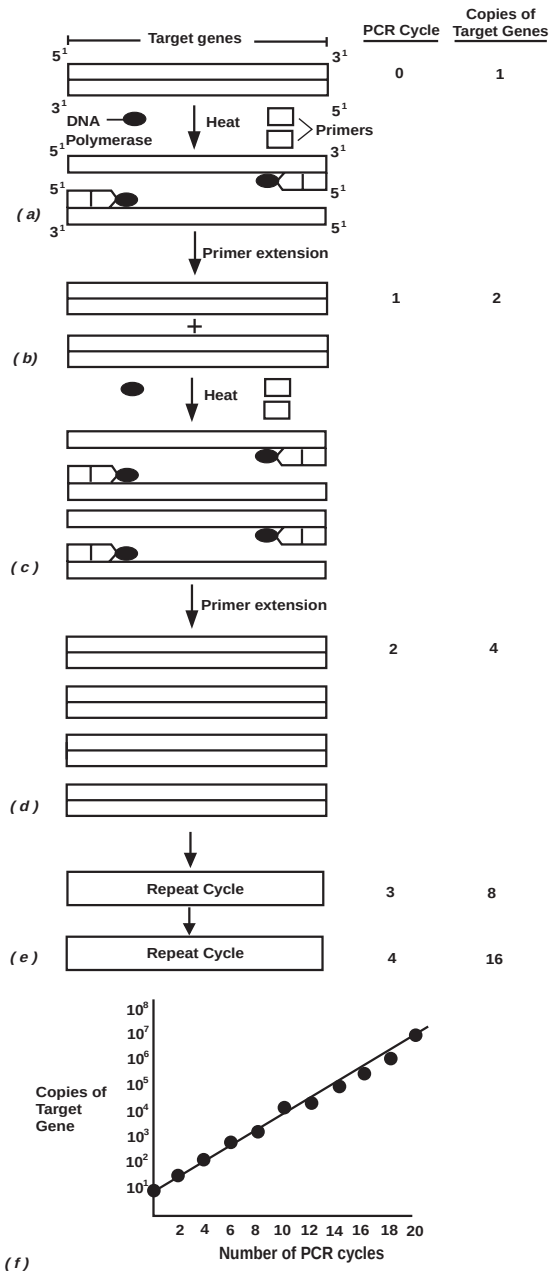

Figure 10-12. Polymerase chain reaction. (a) Target DNA is heated to separate the strands and two primers, one complementary to the target strand and one to the complementary strand, are added along with DNA polymerase. (b) Following reannealing, primer extension yields a copy of the original double-stranded DNA. (c) Further heating, primer addition, and primer extension yields a second double-stranded DNA. (d) The second double-stranded DNA. (e) Two additional PCR cycles yield 8 and 16 copies, respectively, of the original DNA sequence. (f) Effect of running 20 PCR cycles on a DNA preparation originally containing 10 copies of a target gene

tion, control and management of various diseases. For fish and shrimp farmers, it permits fast, widespread, and sensitive screening of virus carriers, and also for early or light infections. The tests can be carried out non-destructively by using body fragments, blood or feces from fish and shrimp tested. PCR can be used to screen both broodstock animals and also larvae before stocking. PCR is rapidly becoming a critical instrument to detect fish and shrimp pathogens.

PCR assay has been widely used in the detection of fish viruses like stripe jack nervous necrosis virus (SJNNV) (Nishizawa et al., 1994), red sea bream iridovirus (RSIV) (Kurita et al., 1998), aquatic birnaviruses (Williams et al., 1999) and shrimp viruses like white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvo virus (HPV), baculovirus penaei (BP), Taura syndrome virus (TSV), yellow head virus (YHV) and baculoviral midgut gland necrosis virus (BMN) (Lightner, 1996; Wongteerasupaya et al., 1997; Lightner and Redman, 1998; Tapay et al., 1999; Hsu et al., 2000; Magbanua et al., 2000). The assay has also been used in the detection of *Vibrio penaeicida* in shrimp (Genmoto et al., 1996; Nakai et al., 1997) *Aeromonas salmonicida* subspecies *salmonicida* (Miyata et al., 1996), *Pasteurella piscicida* (Aoki et al., 1997) and *Lactococcus garvieae* (Aoki et al., 2000) in fish.

PCR uses a thermostable polymerase to produce multiple copies of specific nucleic acid region quickly and exponentially. For example, starting with a single copy of a 1 kb DNA sequence, 10^{11} copies (or 100 ng) of the same sequence can be produced within a few hours. Once the reaction has occurred, a number of methods for identification and characterization of the amplification products are then applicable, of which the simplest is to identify the products according to their size following migration in the agarose gels. For many diagnostic applications, the simple visualization of a PCR product of characteristic size is a significant outcome since it indicates the presence of the target DNA sequence in the original sample.

Basic principle

Each PCR amplification is subdivided into three steps which are repeated in cycles as indicated below (Figure 10-12):

(1) **melting or denaturation** (strand separation) of the double strand DNA (one to several minutes at 94-96°C);

(2) **annealing** of the two primers to opposite DNA strands (one to several minutes at 50-65°C); and

(3) **extension** of the primers by polymerase-mediated nucleotide additions to produce two copies of the original sequence (one to several minutes at 72°C).

During cellular DNA replication, enzymes first unwind and denature the DNA double helix into single strands. After the DNA is denatured, one more event must occur before DNA synthesis may be catalyzed by the DNA polymerase. It must find an area of transition from single stranded to double stranded DNA. At proper temperature typically around 55°C, sufficient primer-target DNA hybridization occurs which leads to the synthesis of complementary strands essential for the amplification step. During PCR, high temperature is used to separate the DNA molecules into single strands, and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to a sequence on the opposite DNA strand at the end of the target region.

Components of a typical PCR	
Tris-HCl (pH 8.3)	20 mM
$MgCl_2$	2.5 mM
KCl	25 mM
dNTPs	50 mM each
Primer 1	20 pmol
Primer 2	20 pmol
Taq polymerase	2.5 units
Template DNA	10-100 ng
Mineral oil	optional

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty cycles, a theoritical amplification factor of one billion is attained.assuming **100% efficiency** during each cycle. The final number of copies of the target sequence is expressed by the formula, $(2^n - 2n)x$, where:

n - number of cycles;
$2n$ - first product obtained after cycle 1 and second products

obtained after cycle 2 with undefined length;
x - number of copies of the original template.

There are factors that act against the process being 100% efficient at each cycle. Their effect is more pronounced in the later cycles of PCR. Normally, the amount of enzyme becomes limiting after 25-30 cycles, which corresponds to about 10^6-fold amplification, due to molar target excess. The enzyme activity also becomes limiting due to thermal denaturation of the enzyme during the process. Another factor is the reannealing of target strands as their concentration increases. The reannealing of target strands then competes with primer annealing.

Two important innovations were responsible for automating PCR. First, a heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus* that lives in hot springs. Hence, the term *Taq* (DNA) polymerase came to be. This enzyme remains active despite repeated heating during many cycles of amplification. Second, thermal cyclers were invented which automatically control the repetitive temperature changes required for PCR.

Following amplification, the PCR products are usually loaded into wells of an agarose gel and electrophoresed. Since PCR amplifications can generate microgram quantities of product, amplified fragments can be visualized easily following staining with a chemical stain such as ethidium bromide. The important point to remember is that the amplification is selective - only the DNA sequence located between the primers is amplified exponentially. The rest of the DNA in the genome is not amplified and remains invisible in the gel.

Primer design

Some simple rules aid in the design of efficient primers:

1. Typical primers are 18 to 28 nucleotides in length having 50 to 60% G + C composition. 2. The calculated melting temperatures for a given primer pair should be balanced. One can use the rule-of-thumb calculation of 2°C for A or T and 4°C for G or C. Depending on the application, melting temperatures between 55°C and 80°C are desired.

3. To prevent self-annealing, primers should not be complementary. This precaution is critical at the extreme 3' ends where any complementarity may lead to considerable primer-dimer formation and reduces the yield of the desired product.

4. Runs (three or more) of C's or G's at the 3' ends of primers may promote mispriming at G + C-rich sequences and should be avoided when possible.

If all else fail, it usually helps to try a different primer pair. A less obvious reason for some primers failing to work is the presence of secondary structure in the template DNA. Software is also available from many commercial and academic sources to assist in the process. Most software packages for DNA sequence analysis now include menus for PCR primer design.

Nested PCR

Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR. Nested PCR can also be performed with one of the first primer pair and a single nested primer. The sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using this method. The specificity is particularly enhanced because this technique almost always **eliminates any spurious nonspecific amplification products**. This is because after the first round of PCR, any nonspecific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity.

RNA (RT) PCR

PCR amplifies DNA sequences. In order to perform PCR on RNA sequences using *Taq* DNA polymerase, the RNA must first be transcribed into a cDNA (complementary DNA) copy of the RNA sequence because *Taq* has limited reverse transcriptase activity. This is called reverse transcription (RT). Thus, RNA amplification is achieved by the reverse transcription-polymerase chain reaction (RT-PCR). There are several different kinds of primers that can be used to make cDNAs, like oligo-dT will prime cDNA synthesis on all polyadenylated RNAs, random-primed cDNA synthesis gives a broad range of cDNAs and is not limited to polyadenylated RNAs and lastly, oligo-nucleotide primers complementary to the RNA(s) of interest may be used to synthesize highly specific cDNAs.

One-tube RT-PCR incorporates both the reverse transcriptase enzyme and a thermostable DNA polymerase in a single tube for synthesis and amplification of the target RNA sequence. This is the preferred procedure for routine analysis. Commercial RT-PCR kits are available and alternatively, reagent mixes can be prepared also from separate component parts.

Problems with PCR

PCR is an extremely powerful technique, but its very power can also lead to considerable problems, particularly when detecting virus or bacterial genes for diagnostic purposes. It is important to remember that nucleic acid from dead as well as viable microorganisms will give a positive reaction. Since even a single molecule of DNA can be amplified by PCR, it is also vital to prevent cross-contamination of DNA samples with amplified or foreign DNA. The slightest contamination of glassware, pipettes or reagents can result in the production of false-positive reactions. Such contamination problems impose a need for extreme cleanliness and rigorous controls. Amplification reactions should be performed in physical isolation (i.e. in a different room) from the parts of the laboratory where specimens are received and target nucleic acid is prepared. Various techniques for reducing extraneous DNA contamination of PCR products have been described, but it is vital that each set of PCR amplifications should include control reactions to verify the purity of reagents and the cleanliness of equipment.

PCR is vulnerable to contamination that will cause erroneous results. False positives will result from contamination of the reaction with target RNA or RT-PCR products. False negatives can be caused by the presence of inhibitors in the test samples or badly degraded target materials. For competent PCR, the sample must be either fresh and in good condition prior to nucleic acid extraction or preserved to maintain nucleic acid suitable for extraction. Proper consideration to the extraction procedure is also important. Maintenance of rigorously clean experimental techniques, use of standard reaction conditions and inclusion of internal standards as positive and negative controls are essential to gain accurate interpretation of the results.

Other problems may arise from the relatively high error rate of *Taq* polymerase. Base substitutions occur at about one in every 9,000 bp, and frameshifts at about one in every 40,000 bp. Although such error rates may seem to be insignificant, they may have profound effects on the homogeneity of the amplified products.

Although PCR can now be semi-automated because of the availability of the thermocycler, the technique still requires a certain amount of technical skill and some specialized equipments to prepare samples and perform amplification reactions successfully.

SUMMARY

The improvement of existing immunoassay techniques, development of monoclonal antibody technology and the development of new immunoassay approaches are all working together to provide new tools for the detection of disease-causing organisms in fish and crustaceans. Following the introduction of nucleic acid hybridization technique and PCR, it was recognized that the methods offered a sensitive approach to the detection and identification of specific microorganisms as in the case of a bacterial or viral infection in a variety of sample types. Potentially, a characteristic DNA sequence from a single virus particle or cell of a particular organism can be amplified to detectable levels within a short period of time. Conventional diagnostic methods that involve the culture of microorganisms can take days or weeks to complete or very tedious to perform. PCR offers a rapid, very sensitive, very specific and simple alternative. Further developments in immunodiagnostics and emerging technologies such as DNA-based tests will revolutionize the detection and identification of infectious disease agents.

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CHAPTER ELEVEN

Harmful and toxic algae

Romeo D. Caturao

Microscopic algae are important food for filter-feeding bivalves (oysters, mussels, scallops and clams) and for the larvae of commercially important crustaceans and fishes. Proliferation (algal blooms) of this algae up to a million cells/l, or cells/ml, is beneficial to aquaculture and wild fisheries operations. In some situations, however, algal blooms can cause severe economic losses to aquaculture, fisheries and tourism, and have major impacts on health and environment. There are 5,000 species of marine phytoplankton (Sournia et al. 1991). Some 300 species can, at times, occur in such high density that they discolor the surface of the sea (red tides). Only 40 species have the capacity to produce potent toxins that can find their way to fish and shellfish and, eventually, to humans.

The first written reference to harmful algal bloom appears in the Bible (1,000 years B.C). In Exodus 7:20-21 is written that at that time in Egypt all the waters in the river turned into blood, and all the fish in the river died, and the river stank, and the Egyptians could not drink the water in the river. In this case, a non-toxic bloom-forming alga became so densely concentrated that it generated anoxic conditions resulting in indiscriminate kills of both fish and invertebrates. Oxygen depletion developed due to high respiration by the algae (at night or in dim light during the day), but most probably, bacterial respiration during decay of the bloom caused it.

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins was in 1793 in Poison Cove in British Columbia. The seawater became phosphorescent due to dinoflagellate blooms. The causative alkaloid toxins, now called paralytic shellfish poisons (PSP) are so potent that about 500 micrograms of toxins, which can easily accumulate in just one serving of shellfish (100 gram), could be fatal to humans.

On a global scale, close to 2,000 cases of human poisoning (15% mortality) through fish or shellfish consumption are reported each year and, if not controlled, the economic damage through reduced local consumption and reduced export of seafood products can be considerable. Whales and porpoises can also become victims when they receive toxins through the food chain via contaminated zooplankton or fish. Poisoning of manatees by dinoflagellate brevetoxins contained in salps attached to seagrass, and of pelicans by diatom domoic acid contained in anchovies have also been reported.

Harmful algal bloom has become apparent only as a result of increased interest in intensive aquaculture systems for finfish. Some algal species can seriously damage fish either mechanically or through production of hemolytic sub-

stances. While wild fish stocks have the freedom to swim away from problem areas, caged fish appears to be extremely vulnerable to such noxious algal blooms.

In the Philippines, red tide was reported in 1908 in Manila Bay as due to *Peridinium* blooms. Thereafter, minor nontoxic red tide outbreaks became almost an annual event in Manila Bay, particularly in the Cavite Area. It was not until June 1983 that the first outbreak of a toxic red tide caused by *Pyrodinium bahamense var. compressa* occurred in Samar, Central Philippines.

In 1987, the presence of dinoflagellate blooms caused by *Pyrodinium bahamense var* was detected in the coastal waters of Masinloc, Zambales, extending from Subic to Santa Cruz. Almost simultaneously at that time, the toxic red tide recurred in Samar. Another case of paralytic shellfish poisoning (PSP) was reported on August 19, 1988 in Orion, Bataan, followed by another 28 cases in Limay within a four-day period.

CONDITIONS THAT STIMULATE HARMFUL AND TOXIC ALGAL BLOOMS

Eutrophication

Eutrophication due to upwelling of deep seawater

Particulates of decayed phytoplankton may sink several meters a day. These particulates are mineralized by bacterial activities before they are deposited to the ocean bed. The nutrients released by decomposition of these particulates can drift below photic zone. For phytoplankton forming red tides, therefore, eutrophication of surface water by supplying nutrients, especially phosphorous is necessary. Eutrophication in the open sea is affected by upwelling whereby transport of deep water to the euphotic zone is driven by a force mainly originated from an eddy, enriching the coastal water column and initiating plankton blooms.

Terrestrial nutrition supplies

This is the amount of plant nutrients introduced with river discharge, effluents from aquaculture farms or a terrestrial run off from heavy rains that might provide enough nutrients to stimulate phytoplankton blooms.

Phosphate release from sediments

The increase in activities of sulfate reducing bacteria due to ascending temperature of bottom water that will lead to the production of large amount of H_2S, liberating $P0_4^{3-}$ from F_e bound P in the sediments, could induce phytoplankton red tides.

Nutrient circulation in surf zones

Surf zones are reasonably broad and shallow, cellular circulation predominates that tend to retain nutrients generated by the macrofauna and interstitial microfauna of the beaches. These nutrients may then cause blooms of surf zone phytoplankton, which in turn serves as food for macrofauna filter feeders. With the perimeters of the circulation cells of the surf zone forming its marine

boundary, beach plus surf zone may be denoted as an ecosystem with surf phytoplankton as the primary producers, beach macrofauna the consumers, and the interstitial fauna the decomposers.

Nitrogen fixation in brackishwater

In brackishwater, N_2 fixation by the blue-green algae takes place. But the fixed N is mostly retained by the nitrogen fixers themselves, forming blooms. N_2 fixation decreased with depth in response to light, although other factors are involved. Rates of fixation decrease concurrently with bloom age, total soluble inorganic nitrogen and salinity. Maximum daily fixation occurs early morning.

Vitamin B_{12}

Unlike most centric diatoms studied, none has an absolute Vitamin B_{12} requirement. However, Vitamin B_{12} (5 mg l^{-1}) stimulates growth of most clones by eliminating or shortening the lag phase and increasing the growth rate. High population densities developed 4-54 days with Vitamin B_{12} present. Several clones grown with Vitamin B_{12} removed more than 80% of the Vitamin from the medium. Clearly, B_{12} is important for quick growth and high density of the centric diatoms.

Prolonged residence time of surface water

Stratification of water

Stratification of water prolongs the residence period of the surface water, allowing enough growth for the red tide algae. But sometimes stratification inhibits growth because of lack of ingress of nutrients from outside. Thus, destratification, in some occasions, favors introduction of nutrients from outside and indirectly causes red tides.

Change of direction of periodic wind

Onshore winds carry offshore surface waters to the coast. The buoyancy of the surface water maintains phytoplankton at the surface for a longer period, causing dense blooms along the coast where terrestrial nutrients have been furnished.

Downward migration of phytoflagellates

Downward migration of phytoflagellates appears essential to maintain red tide blooms in estuarine embayment having intensive tidal flushes.

Grazing pressure/depletion

Grazing pressure due to zooplankton on phytoplankton is regarded as a great deterrent of phytoplankton blooms. Sometimes the grazing pressure by macrozooplankton depletion is exhibited on zooplankton, resulting in zooplankton depletion. Occasionally, zooplankton will avoid certain species and reduce grazing response to *Gymnodinium splendens*. Consequently, such phytoplankton grows massively, forming red tides.

These behavioral responses may help explain formation and persistence of dinoflagellate blooms such as red tides in coastal waters often dominated by diatoms with higher maximum growth rates.

Seeding due to temperature induced germination

In summer, phytoflagellates and diatoms stay temporarily in sediment as resting cells such as hypocites or cysts. In spring and for autumn, their germination is initiated with increase and or decrease in sediment temperature, and their swimming cells appear in the upperlying water.

Adaptation to environmental stress

Lowered salinity stress

Marine phytoflagellates and diatoms grow in marine waters with fairly high salinity and also in brackishwater with considerably low salinity. In coastal waters, estuarine surface waters are enriched with nutrients by river water, resulting in lower salinity and consequent phytoplankton stress. Species which can tolerate the stress may grow utilizing these nutrients and develop into red tide blooms. Table 8-1 shows the tolerance of phytoflagellates and diatoms to lowered salinity stress.

Table 11-1. Tolerance of phytoflagellates and diatoms to lowered salinity stress (from Hallegraef et al. 1995)

Phytoplankton	Scientific name	Optimum range of Salinity ($^0/_{00}$)		
Phytoflagellate	*Masarita rotundata*	24	–	30
	Olisthodiscus luteus	10	–	36
	Prorocentrum micans	27	–	36
Diatom	*Skeletonema costatum*	10	–	40
	Chaetocerso spp.	4.4	–	40
	Phaeodactylum tricornutum	8.8	–	40

Evidently, diatoms can tolerate lower salinity levels more than the dinoflagellates. They exploit this difference in utilizing nutrients in coastal waters. This may account for the succession of dinoflagellate blooms.

Lowered silicate stress

Diatoms can make their frutules with less amounts of silicate than usual when silicate in external media is depleted. Dinoflagellate blooms have so often appeared after diatom blooms that there must be a certain rule of succession between diatoms and dinoflagellates.

Trace metal stress

In waters containing metals in very low concentrations, phytoplankton accumulates these metals 10^3–10^4 times higher than they would in ambient water. This might decrease the grazing pressure of zooplankton on phytoplankton having a high content of heavy metals.

Lowered nutrient stress

Ciliates called *"Mesodinium" rubrum* cause red tide and may die under poor nutritive conditions. However, not all of these ciliates may die, some of them may adapt themselves to the said situation, and settle to the bottom for some time. When there is a high supply of nutrient due to eutrophication of marine waters, these ciliates will utilize the nutrients for their growth and eventually will grow dense.

Increased scientific awareness of toxic species

Reports of harmful algal blooms associated with human illnesses or damage to aquaculture operations are receiving increased attention in newspapers, the electronic media and the scientific literature. As a result, more and more researchers are surveying their local waters for the causative organisms.

Increased utilization of coastal waters for aquaculture

Aquaculture operations act as sensitive bioassay systems for harmful algal species and can bring to light the presence of problem organisms in waters not known to exist before. The increase in shellfish farming worldwide is leading to more reports of paralytic, diarrhetic and neurotoxic or amnesic shellfish poisoning. On the other hand, increased finfish culture is drawing attention to algal species, which can cause damage to delicate gill tissues of fishes.

Increase algal blooms by cultural eutrophication

While some organisms such as the dinoflagellates *Gymnodinium brev*e, A*lexandrium* and *Pyrodinium* appear to be unaffected by coastal nutrient enrichments, many other algal species appear to be stimulated by cultural eutrophication from domestic, industrial and agricultural wastes.

Unusual climatological conditions

The coincidental occurrence of *Pyrodinium* blooms and El Niño-Southern Oscillation (ENSO) climatological events presented strong circumstantial evidence on the possible impacts on algal bloom. El Niño is caused by an imbalance in atmospheric pressure and sea temperature between the eastern and western parts of the Pacific Ocean, which results in a shoaling of the thermocline. The 1991-1994 ENSO event and the recurrence of dinoflagellate blooms in the Philippines tend to substantiate these claims.

Transport of dinoflagellate cysts

Cargo ballast water was first suggested as a vector in the dispersal of non-indigenous marine plankton some 90 years ago. However, in the 1980s the problem of ballast water transport of plankton species gained considerable interest when evidence was brought forward that non-indigenous toxic dinoflagellate species had been introduced into Australian waters including sensitive aquaculture areas, without disastrous consequences for commercial shellfish farm operations. Another vector for the dispersal of algae (especially their resting cysts) is with the translocation of shellfish stocks from one area to another. The feces and digestive tracts of bivalves can be loaded with viable dinoflagellate cells and sometimes can also contain resistant resting cysts.

TYPES OF HARMFUL AND TOXIC ALGAL BLOOMS

Type A species

These are species that produce basically harmless water discoloration; however, under exceptional conditions in sheltered bays, the blooms can grow and cause indiscriminate kills of fish and invertebrates from oxygen depletion. The blooms are represented by dinoflagellate species *(Gonyaulax polygramma, Noctiluca scintillans,* and *Scrippsiella trochoidea)* and *Cyanobacterium (Trichodesmium erythraeum)* (Fig. 11-1).

Type B species

Type B species produce potent toxins that find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses such as:

- Paralytic Shellfish Poisoning (PSP) – Examples: dinoflagellates *(Alexandrium acatenella, A. catenella, A. cohorticula, A. fundyense, A. fraterculus, A. minutum, A. tamarense, Gymnodinium catenatum,* and *Pyrodinium bahamense var. compressum)* (Fig. 11-2a).
- Diarrhetic Shellfish Poisoning (DSP) – Examples: dinoflagellates *(Dinophysis acuta, D. acuminata, D. fortii, D. norvegica, D. mitra, D. rotundata, Prorocentrum lima)* (Fig. 11-2b).
- Neurotoxic Shellfish Poisoning (NSP) – Example: dinoflagellate *(Gymnodinium breve, G. cf. Breve,* New Zealand*)* (Fig. 11-2c).
- Amnesic Shellfish Poisoning (ASP) – Example: diatoms *(Pseudo-nitzschia multiseries, P. pseudodelicatissima,* and *P. australis)* (Fig. 11-2d).
- Ciguatera Fish Poisoning (CFP) – Example: dinoflagellate *(Gambierdiscus toxicus, Osteopsis spp., Prorocentrum spp.)* (Fig. 11-2e).
- Cyanobacterial Toxic Poisoning (CTP) – Example: cyanobacteria *(Anabaena circinalis, Microcystis aeruginosa, Nodularia spumigena)*

Type C species

Species of this type are non-toxic to humans, but harmful to fish and invertebrates (especially in intensive aquaculture systems) by damaging or clogging their gills.

Examples are diatoms *(Chaetoceros convolutus),* dinoflagellate *(Gymnodinium mikimotoi),* prymnesiophytes *(Chrysochromulina polylepsis, Prymnesium parvum, P. patelliferum),* and raphidophytes *(Heterosigma carterae, Chattonella antiqua).*

Figure 11-1. Red tide microalgae (type A – useful, mostly harmless; type B – potentially harmful by oxygen depletion; type C – harmful, responsible for fish mass mortality)

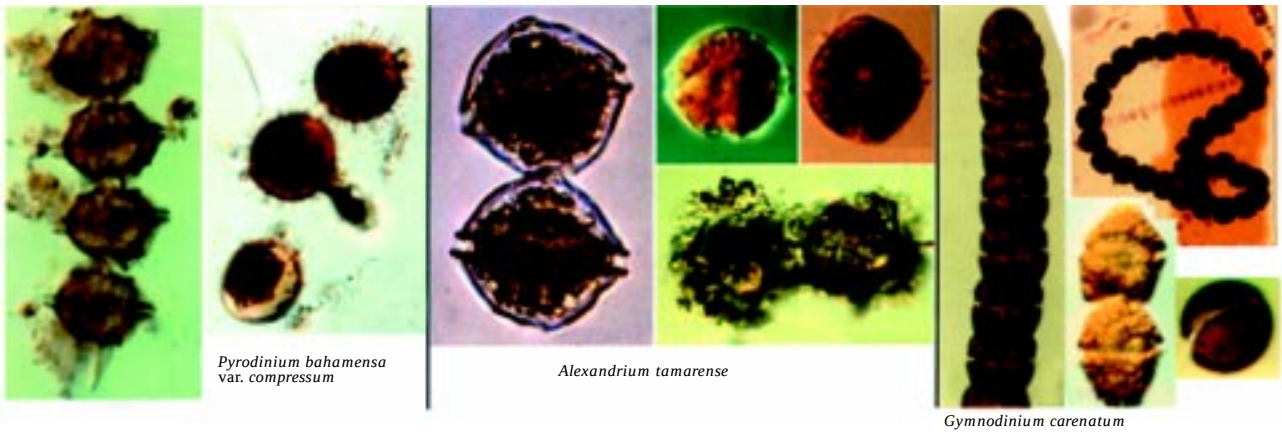

Figure 11-2a. Species responsible for Paralytic Shellfish Poisoning

Figure 11-2b. Species responsible for Diarrhetic Shellfish Poisoning

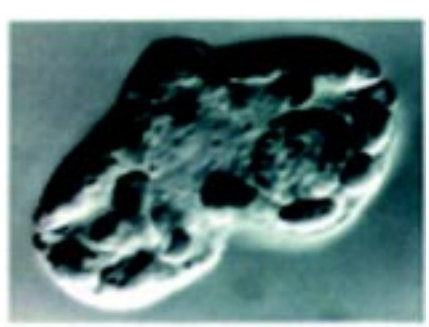

Figure 11-2c. *Gymnodinium breve*, responsible for Neurotoxic Shellfish Poisoning

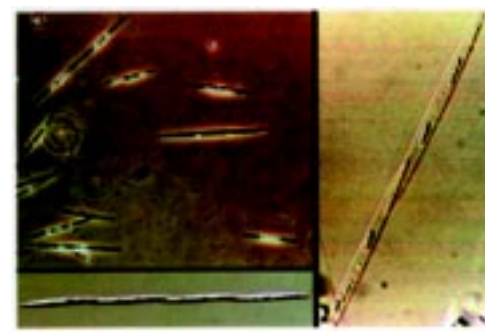

Figure 11-2d (far right). *Pseudonitzschia* spp., responsible for Amnesic Shellfish Poisoning

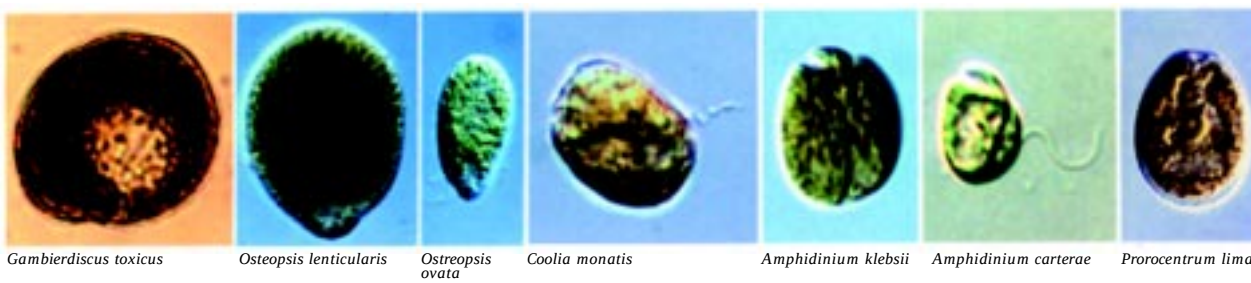

Figure 11-2e. Species responsible for and implicated in Ciguatera Fish Poisoning

EFFECTS OF HARMFUL AND TOXIC ALGAL BLOOMS TO FISH AND MARINE ENVIRONMENT

Red tide-inducing phytoplankton produce many lipids containing highly unsaturated fatty acids along with amino acids. Much carbohydrate is also released during the bloom. Decaying phytoplankton red tides sink slowly receiving heterotrophic decomposition due to bacteria and deposit on the seabed. If waters are shallow with low temperatures, phytoplankton deposits remain intact and benthos can utilize them over long periods. Low temperature of bottom water will cause rapid decomposition, including dissolved oxygen deficiency and the generation of H_2S. This situation will eventually damage the benthos.

Mass destruction of marine resources results from respiratory difficulty as evidenced by mortality of yellowtail *Seriola quinqueradiata* due to *Chatonella antiqua* red tide, and of whitefish *Coregonus lavaretus* due to *Ceratium hirundinella* bloom. Mass mortality of finfish and shellfish have occurred elicited by toxins produced by phytoflagellates and blue-green algae, notably brevetoxins by *Ptychodiscus brevis* and aphatoxin by *Aphanizomenon fros-aqua* blooms.

Phytoplankton that cause red tides affect distribution of heavy metals in marine waters by ion exchange of metals bound to phytoplankton cells. Heavy metals so bound are deposited with phytoplankton on the seabed. Phytoplankton red tide may increase the concentrations of heavy metals like Cd and Fe by producing humic acid. The remarkable sea foam accompanied by unusually high concentrations of dissolved organic matter manifested surfactant production due to a *Phaeocystis pouchetti* red tide. Similar productions were observed by species of *Ballariophyceae*, *Cryptophyceae*, *Haptophyceae* and *Chlorophyceae*. Association of Cu, Hg, and Pb with these surfactants was confirmed. Antimicrobial compounds were demonstrated in *Gonyaulax tamarensis*, which has a broad antimicrobial spectrum. Antimicrobial agents were also found in *Gyrodinium cohnii*, but not in axenic culture.

Toxin-producing algae are becoming an increasingly serious problem in both aquaculture and fisheries populations. The severity of these blooms has appeared to increase in recent years. Likewise, the number of geographic locations experiencing toxic algal blooms has also increased. Toxic algal blooms are worldwide problems affecting aquatic populations in both warm water and cold water environments. The great majority of the problems has occurred in near-coastal marine ecosystems and thus, may have the greatest potential impact on cage and net pen culture. They are also a threat to culturists obtaining water from sites prone to develop toxic algal blooms (e.g., land-based tank systems). In some cases, the introduction of toxic algae into a closed aquaculture system may result in devastating effects.

Increased eutrophication or nutrient enrichment has often been correlated with increased prevalence of many toxic algal blooms. But there appear to be other important risk factors besides simply increased nitrogen or phosphorous, which are essential for development of bloom. The problem of toxic algae is a cogent lesson on how the success of marine aquaculture is heavily dependent

upon a healthy, balanced natural environment and that aquaculturists have a vested interest in assuring that the marine environment is not degraded.

Many different toxic algae have been implicated in fish kills, although in most cases the precise toxins or mechanisms of toxicity are unclear. Nonetheless, many different types of toxins have been isolated from various algae and many of these are ichthyotoxic. Neurotoxins are especially common. Clinical signs of algal neurotoxicity include disorientation, loss of equilibrium, and sporadic hyperactivity. Other algae mechanically obstruct or damage the gills of fishes, causing hypoxia.

Dinoflagellates represent the predominant toxin producing algae. Many dinoflagellates have been implicated or suspected in fish kills. The red tide dinoflagellate (*Gymnodinium breve*) causes mass mortalities of fish and invertebrates in states bordering the Gulf of Mexico. Some dinoflagellate toxins are transferred up the food chain (e.g., *Alexandrium*) and have caused mortalities in wild fish (e.g., Atlantic herring *Clupea harengus*) that consume tainted zooplankton along the northwest Atlantic coast of the United States.

Other algal blooms associated with toxicity include the *Prymnesium parvum*, which causes mortality in brackishwater and marine pond fish (e.g., mullet *Mugil spp*.) in Europe and the Middle East. Diatoms of the genus *Chaetoceros* have been associated with mortality in seawater-cultured salmonids. The spines of other alga apparently cause it to become lodged on or in the gills. Diatoms may become embedded in gill tissue, inciting a foreign body reaction. Epithelial hyperplasia causes hypoxia. In some cases, hyperactive mucus production appears to be primarily responsible for the hypoxia. Other algae have been less commonly associated with ichthyotoxicity, although there is considerable speculation about possible sublethal effects.

The most well recognized economic impact of toxic algae is seafood contamination by their toxins (Table 11-2). This has caused major economic losses in shellfish, including cultured species. Other toxic algae are known to cause serious illness in persons exposed to their toxins. Thus, the presence of toxic algae poses a serious threat to the future success of marine aquaculture, and there is a need to have intensified efforts to address these problems.

The price of fish and invertebrates has dropped as a result of red tide scare, particularly in the Philippines. Prices of fishes caught from other areas free from red tide contamination have also been affected by the red tide scare.

Table 11-2. Causative organisms, clinical signs and potential treatments of various types of fish and shellfish poisoning among human consumers (Hallegraef et al. 1995)

Type	Causative organisms	Symptoms		Treatment
		Mild case	Extreme case	
Paralytic shellfish poisoning (PSP)	*Alexandrium catenella* *Alexandrium minutum* *Alexandrium tamarense* *Gymnodinium catenatum* *Pyrodinium bahamense*	Within 30 min: tingling sensation or numbness around lips, gradually spreading to face and neck; prickly sensation in fingertips and toes; headache, dizziness, nausea, vomiting, and diarrhea	Muscular paralysis; pronounced respiratory difficulty; choking sensation; death through respiratory paralysis may occur within 2-24 h after ingestion	Patient has stomach pumped and is given artificial respiration. No lasting effects
Diarrhetic shellfish poisoning (DSP)	*Dinophysis acuminata* *Dinophysis fortii* *Dinophysis norvegica* *Prorocentrum lima*	After 30 min, to a few h (seldom more than 12 h); diarrhea, nausea, vomiting, abdominal pain	Chronic exposure may promote tumor formation in the digestive system	Recovery after 3 days, irrespective of medical treatment
Amnesic shellfish poisoning (ASP)	*Pseudo-nitzschia multiseries* *Pseudo-nitzschia Pseudodelicatissima* *Pseudo-nitzschia australis*	After 3-5 h; nausea, vomiting, diarrhea, abdominal cramps	Decreased reaction to deep pain; dizziness, hallucinations, confusion; short-term memory loss; seizures	
Neurotoxic shellfish poisoning (NSP)	*Gymnodinium breve; G. cf. breve* (New Zealand)	After 3-6 h; chills, headache, diarrhea, muscle weakness, muscle and joint pain; nausea and vomiting	Paraesthesia: altered perception of hot and cold; difficulty in breathing, double vision, trouble in talking and swallowing	
Ciguatera shellfish poisonig	*Gambierdiscus toxicus* *?Ostreopsis siamensis*	Symptoms develop within 12-24 h of eating fish. Gastrointestinal symptoms; diarrhea, abdominal pain, nausea, and vomiting	Neurological symptoms: numbness and tingling of hands and feet; cold objects feel hot to touch; difficulty in balance; low heart rate and blood pressure; rashes. In extreme cases, death through respiratory failure	No antitoxin or specific treatment is available. Neurological symptoms may last for months and years. Calcium and mannitol may help relieve symptoms

STRATEGIES IN COPING WITH THE PROBLEM OF HARMFUL AND TOXIC ALGAL BLOOMS

Harmful and toxic algal bloom is one of the major public health and fishery problems in the Philippines and throughout the world. Its occurrence can cause serious social problems. The Philippine government, starting in 1984, has adopted some strategies for all red-tide affected areas in the country in an effort to detect a bloom at its early stage and minimize, if not totally eliminate, its effects on public health.

Red Tide Monitoring

Weekly or twice monthly monitoring of plankton and shellfish samples should be collected in coastal areas, particularly with histories of *Pyrodinium* blooms. During red tide blooms, aerial surveillance from helicopters and light aircraft should be undertaken, in coordination with concerned agencies like the Department of Agriculture in the case of the Philippines, to determine the extent of the bloom and the movement of the visible red tide. Based on the information gathered from these aerial observations, the residents in affected areas are to be alerted.

Other hydrobiological parameters such as water temperature, salinity pH, dissolved oxygen, phosphate-phosphorus and cyst density should be determined once a month during neap tides to minimize the tidal effect on the water samples.

Meteorological parameters such as amount of rainfall, wind force and wind direction should be taken from the records of the Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA), in the case of the Philippines.

Information Dissemination

Information dissemination on red tide to alert the public through print and broadcast media, including seminars and meetings, must be done whenever the toxicity in shellfish exceeds the regulatory limit of 80 microgram toxin per 100 g of shellfish meat. During the emergency, the people should be advised not to eat any kind of shellfish taken from red tide-infested waters.

Regulation

A temporary ban on the harvesting, marketing and transporting of all kinds of marine shellfish from the red tide contaminated waters should be imposed upon information that the toxin level in shellfishes has exceeded the regulatory limit. Issuance of auxiliary invoices, a requirement in transporting fishery products from one place to another, should be suspended to prevent movement of the contaminated shellfish to non-affected areas. This should be matched by establishing checkpoints in strategic locations such as piers, airports and bus routes.

Medical Management of PSP Cases

There is no antidote for the PSP toxin. Treatment is largely symptomatic, as indicated by Halstead (1965). In the Philippines, as mentioned by Gonzales (1989), it is suggested that the first thing to consider in PSP management is to empty the stomach of the victim of the toxic material as quickly as possible. This may be done by giving the patient an oral emetic, or, when this is not available, by inserting a finger in the throat of the victim to induce vomiting. Since the toxin is water soluble, it is also recommended that the victim be given plenty of water to induce urination and minimize absorption of the toxin through the gastro-intestinal tract.

The Department of Health and the Philippine General Hospital follow a protocol in managing PSP cases as cited by Gonzales (1986). The universal antidote for all kinds of poisoning contracted through the oral route is pure coconut milk. Gacutan (1986) suggested that this local cure for PSP is effective. However, it should be emphasized, that coconut milk, or any drug for that matter, should not be given to patients starting to show symptoms of dysphagia and respiratory failure due to the risk of asphyxia.

Government Assistance Program

The occurrence of red tide, and the red tide scare itself, will adversely affect the income of fishermen and fish vendors, while others may not even earn a single centavo at all. Due to economic hardship suffered by fishermen during red tide occurrences, the government, through non-government organizations and fishermen's cooperatives, should grant red tide affected artisanal fishermen with emergency loans payable within a period of one year with affordable interest rate.

Recommendations

Efforts should be made to collect shellfish at regular intervals, e.g., weekly and subject this to mouse bioassay. Also, water samples must be collected weekly and analyzed for the presence of harmful and toxic algal blooms by the Bureau of Fisheries and Aquatic Resources. Efforts must also be exerted to develop the regional and provincial capabilities of the Department of Agriculture and the Department of Health on bioassay techniques to determine the presence or absence of toxin in shellfish samples. If PSP toxins are detected from the sample, a warning may be issued while waiting for the results of the confirmatory tests to be performed on the samples by the Bureau of Food and Drugs. This way, the government may be able to respond quickly should there be a recurrence of toxic red tide.

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GLOSSARY

abscess – a collection of pus in a body cavity formed by tissue disintegration

acute – rapid and severe in development

aflatoxicosis – the disease resulting from exposure of animals to aflatoxin-contaminated diets

aflatoxin – a highly toxic substance produced by fungi growing on feed or feed ingredients

agglutination – reaction between antibody and cell-bound antigen resulting in clumping of the cells

amino acid – basic component of protein

amnesic shellfish poisoning (ASP) – a phycotoxic syndrome characterized by memory loss with extreme cases of human intoxication from shellfish contaminated by domoic acid (DA).

anchor – used for attachment to the host

anchoring disc – a laminar structure at the anterior end of a microsporean spore

anemia – a condition characterized by a deficiency of hemoglobin, packed cell volume, or erythrocytes in the blood

anorexia – loss of appetite

antibody – a glycoprotein produced in response to the introduction of an antigen; it has the ability to combine with the antigen that stimulated its production. Also known as immunoglobulin.

antigen – a foreign (nonself) substance (such as a protein, nucleoprotein, polysaccharide or sometimes a glycolipid) to which lymphocytes respond; also known as immunogen because it induces the immune response

antioxidant – a substance that chemically protects other compound against oxidation; for example, vitamin E prevents oxidation and rancidity of fats

antiserum – serum containing induced antibodies

asexual – reproduction not involving karyogamy or meiosis.

ascites – the accumulation of serum-like fluid in the abdomen.

asphyxiation – deficiency of oxygen

atrophy – a degeneration or diminution of a cell or body part due to disuse, defect, or nutritional deficiency

axenic – without another organism being present; pure culture

backwash – to clean by reversing water flow

bacteria – one-celled microorganisms which lack well-defined nucleus

bioassay – biological assay; a procedure involving the use of responses of aquatic organisms to detect or measure the presence or effect of one or more substances

biotroph – refer to obligate parasite.

blister – a thin vesicle, especially on the skin, containing watery matter

carotenoid – a class of pigments occurring in the tissues of algae and higher plants

carrier – one that transmits disease germs

cartridge filter – tubular filter device made up of spun polypropylene material inserted inside a filter housing case

cataract – development of partial or complete opacity of the crystalline lens of the eye or its capsule.

cercariae – free-swimming larval stage of digeneans

chemotherapy – use of a specific chemical agent to control a disease

chronic – lingering

ciguatera fish poisoning (CSP) – a seafood poisoning which results from consumption of tropical reef fish contaminated by ciguatoxins and characterized by a wide array of gastrointestinal and neurological symptoms

ciguatoxins (CTX) – a kind of toxin produced by benthic dinoflagellates found in corals. It is a family of complex lipid-soluble, highly oxygenated cyclic polyether compounds

cilia – short, hair-like structures used for movement

clonal selection theory – the theory that clones of effector B and T cells arise from single cells (or very small clones) that are stimulated to reproduce by antigen binding to their receptors

clone – a group of genetically identical cells or organisms derived by asexual reproduction from a single parent

coccon – a brooding capsule for leech eggs

coelozoic parasites – parasites which occur in organ cavities

coenocytic – nonseptate; referring to the fact that nuclei are present in the cytoplasm without being separated by cross-walls; the nuclei lie in a common matrix.

commensalism – a relationship wherein one organism lives and derives benefit on another organism without causing any harm

complementary – nonidentical but related genetic structures that show precise base pairing

conidiophore – a simple or branched hypha arising from a somatic hypha and bearing at its tip or side one or more conidiogenous cells.

conidium (pl. conidia) – a non-motile asexual spore usually formed at the tip or side of a sporogenous cell.

copepods – small planktonic or bottom dwelling crustaceans

cross-contamination – transmission of disease or disease agent from one tank to another

cutaneous lesion – lesion on the skin

cyanobacterial toxic poisoning (CTP) – poisoning due to cyanobacteria which produce a diverse range of secondary metabolites including hepatotoxins, neurotoxins and cytotoxins

cyst – a non-motile, resistant, dormant stage

debris – organic waste from dead cells or unused food

definitive host – the host in which the parasite undergoes sexual reproduction

denaturation – a change in the shape of an enzyme that destroys its activity; the term is also applied to changes in nucleic acid shape

deoxyribonucleic acid (DNA**) –** the nucleic acid that constitutes the genetic material of all cellular organisms. It is a polynucleotide composed of deoxyribonucleotides connected by phosphodiester bonds

diagnosis – the act of identifying the cause of disease

diarrhetic shellfish poisoning (DSP) – a complex gastro-intestinal syndrome associated with the consumption of contaminated shellfish by humans

dinoflagellates – are group of unicellular eukaryotic organisms which swims by means of a pair of whip-like flagella

disease – an abnormal occurrence displayed by living organisms through a specified common characteristic (symptom), or set of characteristics

DNA polymerase – an enzyme that synthesizes new DNA using a parental DNA strand as a template

domoic acid (DA) – a naturally occurring compound belonging to kainoid class of compounds that has been isolated from a variety of marine sources including macro and micro-algae

dropsy – ascites; abnormal accumulation of liquid in internal organs or tissues

DSP toxins (DSPTX) – are liquid-soluble long chain compounds containing tans-fused or spiro-linked cyclic polyether rings

dystrophy – abnormal development or degeneration

ectoparasite – parasite living in the external surfaces of the host

edema – excessive accumulation of fluid in tissue spaces

electrophoresis – a technique that separates substances through differences in their migration rate in an electric field due to variations in the number and kinds of charged groups they have

emaciation – become abnormally lean

encapsulation – the covering of a parasite by the host

encystment – the covering of a parasite with materials of parasite origin

endemic – recurring in a locality

endoparasite – parasites living inside the body of the host

endospore – the thick inner chitinous layer of the wall in a microsporean spore

endotoxin – a toxin of internal origin present in bacteria but separable from the cell body only on its disintegration

enteritis – the inflammation of the intestine

enzyme – a protein catalyst with specificity for both the reaction catalyzed and its substrates

Enzyme-linked Immunosorbent Assay (ELISA) – a technique used for detecting and quantifying specific antibodies and antigens

epizootic – widespread outbreak of fish diseases

etiology – the study of the cause of disease

exophthalmia – abnormal protrusion of the eyeball

exopthalmus – abnormal protrusion of the eye.

exospore – the proteinaceous outer layer of the wall in a microsporean spore

exotoxin – a soluble toxin that passes into the medium during growth of certain bacilli or other microorganism

facultative parasite – a saprobic organism capable of infecting another living organism under some conditions

facultative saprobe – a parasitic organism capable of growing on dead organic matter under some conditions

fibrosis – inflammatory response; in shrimp hepatopancreas, characterized by the presence of connective tissues in between the tubules

flagella – long, hair-like structures used for locomotion

formalin – a 37% solution of formaldehyde

fungus – a general term for a group of eukaryotic protista (e.g., mushrooms, yeasts, molds etc.) marked by the absence of chlorophyll and the presence of a rigid cell wall

gel – an inert polymer, usually made of agarose or polyacrylamide, used for separating macromolecules such as nucleic acids or proteins by electrophoresis

gene – a unit of heredity; a DNA segment or sequence that codes for a polypeptide, rRNA or tRNA

genetic manipulation – selective breeding of organisms that are resistant to disease or those that grow larger or faster

genome – the full set of genes present in a cell or virus; all the genetic material in an organism.

halophilic – a microorganism whose growth is accelerated or dependent on high salt concentration

hemocyanin – a colorless copper-containing respiratory pigment found in solution in the blood plasma of various arthropods and molluscs

hemocyte – a blood cell

hemocytic infiltration – inflammatory response; in shrimp hepatopancreas, characterized by presence of hemocytes in between the tubules

hemoglobin – the respiratory pigment of red blood cells that takes up oxygen at the gills or lungs and releases it to tissues

hemorrhage – discharge of blood from a ruptured blood vessel

hemorrhage – internal bleeding and subsequent clotting caused by the rupture of blood vessels

hepatopancreas – a glandular structure in animals that functions in digestion, absorption, and storage

heterotrophs – organisms which use organic compounds as primary source of energy

holdfast – the attachment organ of some parasites

holocarpic – having all the thallus used for the fruit body.

homeostasis – a condition whereby animals maintain a relatively stable internal environment

host – a living organism harboring another organism

hyaline – transparent or transluscent.

hybridization – the natural formation or artificial construction of stable hybrid nucleic acid complexes by complementary base pairing between two nucleic acid strands

hybridoma – the fusion of a malignant cell with a single B-lymphocyte to produce a malignant lymphocyte producing monoclonal antibody

hyperemia – abnormal accumulation of blood in any part of the body

hyperplasia – an abnormal or unusual increase in the number of cells of a tissue

hypertrophy – an increase in size of a tissue or an organ due to an increase in size of individual cells

hypha – the unit of structure of most fungi; a tubular filament.

immunization – process that equip an organism with antibodies effective against specific pathogens, by provoking their manufacture in the absence of infection

incubation period – period from entry of a pathogen to the appearance of the first signs of disease

infection – a pathological condition due to the growth of microorganisms in a host

infestation – a pathological condition due to the presence of parasites

inflammation – a tissue reaction resulting from an irritation by a foreign material and causing a migration of leukocytes and increased flow of blood to the area, producing swelling, reddening, heat, pain and tenderness

ischemia – localized tissue anemia due to obstruction of the inflow of blood

intermediate host – a host in which the larval stages of a parasite develop

intracellular – situated or occurring inside a cell

karyomastigont – the unit of mastigonts and associated nucleus

kinetoplast – the part of the mitochondrion which contains DNA; located near the base of the flagellum and is conspicuous after staining

LC_{50} – medial lethal concentration

lesions – any morbid change in function or structure of an organ or tissue

lethargy – weakness or sluggishness

lordosis – the abnormal forward curvature of the vertebral column

macronucleus – a large nucleus in ciliates which controls trophic functions

mastigont – a complex of flagella-associated organelles in flagellated protozoans, including basal bodies (also called kinetosomes) and projecting and trailing flagella

melanization – inflammatory response characterized by the abnormal accumulation of melanin

melanized – abnormal development of dark coloring matter in the skin

metacercariae – encysted cercariae of digeneans

monoclonal antibody – an antibody of a single type that is produced by a population of genetically identical plasma cells (a clone); produced from a cell culture derived from the fusion product of a cancer cell and an antibody producing cell (a hybridoma)

muscular dystrophy – degenerative disease of the muscles

mutualism – a relationship wherein two organisms benefit from each other

mycelium – mass of hyphae constituting the body (thallus) of a fungus

mycosis (pl. mycoses) – a fungus infection of an animal.

myeloma cell – a malignant tumor of a plasma cell that produces large quantities of antibodies

myopathy – degeneration or atrophy of the muscles

necrosis – localized death of a tissue

necrotized – dead or decayed

neurotoxic shellfish poisoning (NSP) – shellfish poisoning caused by polyether brevetoxins produced by the unarmoured dinoflagellates *Gymnodinium breve*

neurotoxin – toxin that affects the nervous system

Northern Blot – hybridization of a single strand of nucleic acid (DNA or RNA) to RNA fragments immobilized on a filter

nucleic acid hybridization – the process of forming a hybrid double-stranded DNA molecule using a heated mixture of single-stranded DNAs from two different sources; if the sequences are fairly complimentary, stable hybrids will form

nucleotide – a monomeric unit of nucleic acid, consisting of a sugar, phosphate and nitrogenous base

obligate parasite – an organism that can, in nature, obtain food only from living protoplasm; organisms considered as obligate parasites usually cannot be grown in culture or non-living media

oncomiracidium – the free-swimming stage of monogeneans

oviparous – an organism that lays eggs

paralytic shellfish poisoning (PSP) – a neurotoxic syndrome resulting primarily from the blockage of neuronal and mascular Na^+ channels

parasite – an organism that lives at the expense of another, usually invading it and causing disease.

parasitism – a one-way relationship in which a smaller organism (parasite) depends upon and benefits from the bigger (host) organism

paratenic host – a transport host in which the larval stage of a parasite undergoes no development and its only function is to transfer the parasite to the next host

parenteral – injection method of administering drugs

pathogen – a disease-producing agent

pathogenic – capable of producing disease

polar capsule – a thick-walled vesicle in myxosporeans with an inverted polar filament

Polymerase Chain Reaction (PCR**) –** an enzymic method for amplifying exponentially specific preselected fragment of DNA; an *in vitro* technique used to synthesize large quantities of specific nucleotide sequences from small amounts of DNA. It employs oligonucleotide primers complementary to specific sequences in the target gene and special heat-stable DNA polymerases

ppm – parts per million or milligrams per liter or grams per ton

ppt – parts per thousand

precipitation – a reaction between antibody and soluble antigen resulting in a visible mass of antibody-antigen complexes

primer – a short stretch of RNA or DNA used as a starting point for nucleic acid synthesis

probe – a short, labeled nucleic acid segment complementary in base sequence to part of another nucleic acid, which is used to identify or isolate the particular nucleic acid from a mixture through its ability to bind specifically with the target nucleic acid

probiotics – bacteria that promote the growth of an organism or inhibit pathogenic microorganisms

proboscis – a muscular, protrusible feeding organ in some parasitic organisms

procercoid – the first larval stage of many cestodes which develop inside the body cavity of the invertebrate (first) intermediate host

prokaryotic cells – a type of cell whose nuclear substance is not enclosed within a membrane

prophylaxis – preventive action

punctuate – covered or studded with dots, points or minute depressions

quarantine – isolation of material or animal to prevent the spread of infectious disease it carries

rancidity – a condition taking place when the lipids are broken to their constituents through poor storage condition

red tides – reddish-brownish discoloration of the water brought about by sporadic increases in minute or microscopic marine organisms in high density

residual – remaining

ribonucleic acid (RNA**) –** a polynucleotide composed of ribonucleotides joined by phosphodiester bridges

saxitoxin (STX) – a dibasic salt that is very soluble in water and is the most potent neurotoxin found in dinoflagellate blooms

scolex – attachment organelle of some parasites

scoliosis – lateral curvature of the spine

secondary zoospore – kidney-shaped zoospore produced in Oomycota; the flagella are inserted laterally on the spore.

septate – with more or less regularly occuring cross walls.

septicemia – a systemic disease caused by the invasion and multiplication of pathogenic microorganisms in the blood stream

septum (pl. septa) – a cross-wall in a hypha that develops cetripetally.

serological method – process which deals with antigen-antibody reaction

serology – the study of antigen-antibody reactions *in vitro*

shell valve – one of the parts of the myxosporean spore wall

soma – the body of an organism as distinguished from its reproductive organs or reproductive phase.

Southern Blot – the procedure used to isolate and identify DNA fragments from a complex mixture. The isolated, denatured fragments are transferred from an agarose electrophoretic gel to a nitrocellulose filter and identified by hybridization with probes

sporangiophore – a specialized hypha that bears a sporangium.

sporangium – a sac-like structure, the entire protoplasmic contents of which become converted into an indefinite number of spores.

spore – a minute propagative unit functioning as a seed, but differing from it in that a spore does not contain a pre-formed embryo.

spore – the infective stage of an organism that is usually protected from the environment by one or more protective membranes

sporoplasm – the infectious component in spores

stigma – a pigmented red spot in flagellates

stramenopila – a new Kingdom to accommodate most organisms previously referred to the Kingdom Chromista and primarily characterized by the presence of tripartite tubular hairs on flagella or cysts.

straminipilous organisms – bearing tripartite tubular hairs; applicable to flagella and/or cells, whether uniflagellate, multiflagellate or non-flagellate (e.g. cysts)

stress – a factor of an environmental challenge that is severe enough to require a physiological response on the part of the fish

susceptible – disposition to disease

symbiosis – a condition in which two organisms live together

symptom – a physical or physiological change which is caused by a particular disease

thrombosis – the formation of presence of a blood clot within a blood vessel

titer – reciprocal of the highest dilution of an antiserum that gives a positive reaction in the test being used

tomites – cells within the tomont which result from serial binary division

tomont – a cyst-like structure formed by the trophont following detachment from the host

toxin – a poison

trophont – the feeding and growing stage of a parasitic protozoan which differentiates into the reproductive tomont following detachment

ulceration – an open sore on an external or internal surface of the body, usually accompanied by disintegration of tissue with the formation of pus

UV – ultraviolet radiation

vaccination – the process of introducing a suspension of disease-producing microorganisms modified by killing or attenuation so that it will not cause disease and can stimulate the formation of antibodies upon inoculation.

vacuolated – containing spaces or cavities in the cytoplasm of a cell

vector – any agent that transmits an infectious organism

v**irus** – a minute infectious agent which can be resolved or viewed clearly only under a high-powered microscope. It lacks independent metabolism, and is able to replicate only within a living cell

vitamins – an organic compound occurring in minute amounts in foods and essential for numerous metabolic reaction in animals

viviparous – the bearing of live young

zoospore – a motile, asexually produced spore

PHOTO CREDITS

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Representing the Member Countries is the Council of Directors, the policy-making body of SEAFDEC. The chief administrator of SEAFDEC is the Secretary-General whose office, the Secretariat, is based in Bangkok, Thailand.

Created to develop fishery potentials in the region in response to the global food crises, SEAFDEC undertakes research on appropriate fishery technologies, trains fisheries and aquaculture technicians, and disseminates fisheries and aquaculture information. Four departments were established to pursue the objectives of SEAFDEC.

- The Training Department (TD) in Samut Prakan, Thailand, established in 1967 for marine capture fisheries training
- The Marine Fisheries Research Department (MFRD) in Singapore, established in 1967 for fishery post-harvest technology
- The Aquaculture Department (AQD) in Tigbauan, Iloilo, Philippines, established in July 1973 for aquaculture research and development
- The Marine Fishery Resources Development and Management Department (MFRDMD) in Kuala Terengganu, Malaysia, established in 1992 for the development and management of the marine fishery resources in the exclusive economic zones (EEZs) of SEAFDEC Member Countries.

SEAFDEC/AQD is mandated to:

- promote and undertake aquaculture research that is relevant and appropriate for the region
- develop human resources for the region
- disseminate and exchange information on aquaculture

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