STANDARD OPERATING PROCEDURES

for Aquatic Animal Health Management

AQUATIC ANIMAL HEALTH MANAGEMENT

GOVERNMENT OF KERALA

DEPARTMENT OF FISHERIES

THIRUVANANTHAPURAM

STANDARD OPERATING PROCEDURES FOR AQUATIC ANIMAL HEALTH MANAGEMENT

by

Department of Fisheries

Government of Kerala

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Any views or directions expressed in this book are not of the institutes where the authors belongs but of the Department of Fisheries, Government of Kerala.

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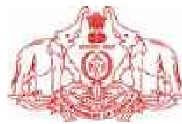

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MESSAGE

01.06.2020

I am happy to know that Department of Fisheries is publishing a Standard Operating Procedure (SO) for Aquatic Animal Health Management. In view of decreasing catch from marine sector, Kerala offers immense scope for fish production through aquaculture development. The state has a deficit of 1.5 lakh metric tones of fish, which is being compensated by import from other states. In the wake of covid -19 and the increasing reports of adulteration in fishes from other states, it is absolutely necessary to achieve self sufficiency in domestic fish production. Many hurdles have to be overcome for attaining this goal. One such problem is the outbreak of diseases in fishes due to the poor quality of seed arrival from other states and unscientific health management. Heavy losses due to disease outbreaks may cause reluctance in taking up aquaculture and as such in hinders the growth of sector. High intensive fish culture systems often possess risks of diseases and hence scientific methods for prevention, surveillance and treatment protocols are necessary for managing disease outbreaks. Expert technical hands along with well established protocols are necessary for handling such diseases and preventing loss to farmers. It is understood that this SOP is prepared through exhaustive discussions with experts of scientific communities from central institutes, the academicians from Kerala University for Fisheries and Ocean Studies (KUFOS) and the officers of the Department who are well experienced in various field conditions of infected fish handling and management. The book will definitely help to manage the commonly encountered fish diseases. It also presents how disease monitoring, surveillance, diagnosis and quarantine can be performed in order to give a base for disease related information system and health certification. This book will provide an extra support for technical hands of the department in aquatic animal health management. I hope that aquatic animal health management will become an easier task for departmental officers in future and thereby self sufficiency in fish production can be achieved in the state in a sustainable manner.

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FOREWORD

Fisheries is the fastest growing food producing sector and can be a solution for deficit in sustainable nutritional food production. Disease is universally recognized as one of the most serious threats to commercial success of aquaculture. Fish pathology has vital importance to fish farming activities, by virtue of the capital which is invested in the same. In developing policies and programmes and in allocating the necessary resources, we are focusing on these areas which has greatest need.

This SOP is the result of exhaustive collaboration between department of fisheries and the research scientists with many years experience in the field of fish pathology. It is a reference guide for the diagnosis of infectious diseases, and a hand book for clinical practitioners and other personnel working in fish health services. Its objective is to present in a concise and clear manner the most important facets of the subject.

The manual has been prepared to provide an overview of basic guidelines to be followed which is easy to handle and understand, not only to the specialist but also for aquaculturists. This will serve as a resource for the identification and control of fish diseases in a multitude of settings, from aquarium fish, to home ponds, to aquaculture species, to fish research and to even wild fish populations.

It is a matter of delight that this is the first SOP for managing diseases of cultured fish and shrimps published at Government level in a state in India. I acknowledge the Directors of CMFRI and CIFT, two leading institutes in the field of fisheries research in India and Vice- Chancellor, KUFOS, the first Fisheries University in India, for providing technical and personnel support in developing this SOP and I wish success for all such future endeavors.

Vikasbhavan,
29-05-2020

Venkatesapathy S. IAS
Director of Fisheries

PREFACE

Kerala is endowed with abundant inland water resources like rivers, rivulets, streams, estuaries and backwaters which are well known for their biodiversity. The annual per capita consumption of fish in Kerala is very high (18.5 kg) compared to the national average (5 kg). As production from capture fisheries is stagnated, aquaculture can be a contributor for sustainable fish production.

The present era is witnessing intensive aquaculture practices such as biofloc farming, re-circuatory aquaculture systems, aquaponics, cage culture etc. in which the fish are exposed to various stressors that are unavoidable components of the culture environment. The intensive phase of development of aquaculture is accompanied with increasing numbers of disease occurrence and awareness about the quality of fish produced from aquaculture facilities. Due to the complex character of fish diseases, solutions are also complex where all elements of the fish-environment-pathogens should be taken into account. A collaborative approach consisting of scientists, extension personnel and the department was required for formulating a standard operating procedure for various aspects of aquatic animal disease and health management. This manual has been drafted based upon inputs from the expert panel of contributors at various workshops.

The book provides meticulous, yet concise descriptions of viral, bacterial, fungal, parasitic and non-infectious diseases in an exhaustive number of fish and shrimp species. The book is designed as a comprehensive guide to the identification and treatment of both common and rare problems encountered in culture conditions. It is based on the methods currently followed by fish pathologists and laboratories and also includes early prevention, detection and treatment. The chapters cover essential information on the basic principles of disease causation, major diseases of cultured fish and shrimp, and standard operating procedures for prevention, control, diagnosis, treatment and quarantine. Health certification forms the key element of such control measures.

We hope that this book would be of immense use to clinical practitioners as well as for the students and academicians as a practical guide and also in stimulating further interest in various diverse fields related to Aquatic animal health management.

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I. INTRODUCTION

Fish production from inland sector is increasing when compared to marine fish production. The overexploitation of marine fish resources, climate change and global warming are having a cumulative effect on these resources. But the increasing demand for fish and the decreasing marine catch has put an excess pressure on aquaculture for intensification. Even though the utilization of water bodies and farm areas for culture has increased there is still a lacuna in production and consumption leading to indiscriminate use of resources and unscientific practices as part of this intensification process. Enhancement of aquaculture production through intensification result in an increased level of stress in aquatic animals from high stocking density and use of chemicals and growth enhancers will ultimately result in diseases. As vertical expansion of fish culture with diversified species and higher stocking density has resulted in more frequent occurrence of bacterial, parasitic and viral pathogens. In aquatic systems, disease management is a difficult proposition due to the unique ecosystem, where the pathogen is always looking for an opportunity when the health status of the host is compromised.

Aquatic animal health management is an important aspect in the light of this intensification process. The unscientific management practices as part of species diversification, intensification etc are the root causes of disease outbreaks. The unethical practice of introduction of exotic species, trans-boundary movement of seeds and adults will also result in introduction of new pathogens. Pathogenic diseases are mainly caused by bacteria, virus, fungi and parasites. Apart from this, nutritional and genetic diseases are also encountered in aquatic animals. Aquatic animal diseases are often problematic and challenging in diagnosis as well as treatment. This SOP for Kerala has been developed to assist the Government through the State Fisheries Department in developing and implementing effective long-term policy and planning for protecting and improving aquatic animal health status. Implementation of the SOP is expected to help us meet international aquatic animal health standards and obligations for the prevention of serious aquatic animal diseases within our territory

The economic losses due to diseases in Indian aquaculture are not properly estimated. However, a few sporadic survey reports are indicative of disease related losses to the Indian aquaculture sector. An earlier survey confined to Andhra Pradesh, India, indicated an annual loss of Rs. 40 million due to diseases. The same

study found that an average of 10% of the production cost was spent on disease treatment. Further, the study recorded disease incidences of parasitic, bacterial and fungal infections being 70%, 27.5% and 2.5%, respectively in freshwater aquaculture. A loss to the tune of Rs 29,524.40 (US$ 615) per ha per year due to argulosis, an ectoparasitic disease from carp culture ponds, was also recorded in a study conducted during 2008-2011. The annual economic losses due to shrimp diseases have been estimated to be Rs. 1022 crores and the losses only due to white spot disease have been reported to be around Rs. 700-800 crores. (National Academy of Agricultural sciences, NAAS, 2019)

Rural resource poor farmers with little or no knowledge of fish health management and skill to prevent and control disease outbreaks are the most sufferers, incurring huge economic loss. The occurrence of disease has become a primary constraint to sustainable aquaculture production and product trade, there by affecting the socioeconomic status of fishers. In Tropical conditions, prompt diagnosis of diseases is necessary because chances of spreading of infections is more. It necessitates well equipped lab to identify diseases in emergency situations and to find out the possible etiology of diseases. It will assist in promoting the sustainable development of the national aquaculture sector and facilitate access to international markets for aquaculture products. Thus, it will protect existing aquaculture, capture and sport fisheries and natural aquatic systems and those who rely upon them, from the social, economic and ecological impacts that can result from aquatic animal disease outbreaks.

II. DISEASE SURVEILLANCE AND REPORTING

Aquatic environment is always dynamic with changing physical, chemical and biological characteristics. Degradation of water quality combined with opportunistic pathogens is often responsible for disease outbreaks. A fish becomes susceptible to disease by any infectious agents, nutritional imbalances and due to environmental stress. Monitoring of temperature, , dissolved oxygen, ammonia, pH, hydrogen sulphide, suspended solids, and alkalinity of the water is essential to ensure that it remains within optimum level so that there is no environmental stress.

Effective surveillance programme is essential to ensure early detection and quick response for controlling further outbreak of diseases. Surveillance and reporting of aquatic animal diseases rest with various levels viz. the farmer, clinical practitioner, laboratory level, district level and state level. When a disease problem develops, a quick and effective response is essential from all of these levels.

1. FARMER LEVEL

1.1. RESPONSE FOR MORTALITY LESS THAN 0.3% PER DAY

- Check for water quality parameters;
- Observe for any unnatural movement or swimming pattern or other behavior;
- Examine the feed intake;
- Record in farm health card.

1.2. RESPONSE FOR MORTALITY BETWEEN 0.3% AND 0.5% PER DAY

- Check for water quality twice daily;
- Observe repeatedly for any unnatural movement or other behavior;
- Examine the fish physically for any deformity;
- Examine feed intake and regulate it accordingly;
- Report to the clinical practitioner within 24 hours;
- Record in farm health card.

1.3. RESPONSE FOR MORTALITY EXCEEDING 0.5% PER DAY

- Report to the clinical practitioner within no time;
- Close the inlet and outlet points of water in the farm;
- Stop feeding and all other activities of the farm;
- Shift the remaining fish to another spare pond or tank, if possible;
- Observe continuously for any unnatural movement or behavioral changes or mortality of animal;

- Collect sample and send directly to the laboratory from the farm;
- Do not destroy the dead fish until sufficient samples are collected for investigation;
- Wait for diagnosis and further instruction from the clinical practitioner;
- Do not sell/ discard any fish without the consent of the clinical practitioner.

2. CLINICAL PRACTITIONER LEVEL

2.1. PASSIVE SURVEILLANCE (in the case of suspected disease/ outbreak)

- Must visit the site within minimum time & carry out disease investigation;
- Observe the farm and premises for any kind of disease outbreak source;
- Refer farm health card;
- Report immediately to district level team, if any infection is suspected;
- Ensure that there is no discharge of water to open source;
- Ensure that feeding and all other farm activities are stopped;
- Ensure that no fish is sold/ discarded without consent of the clinical practitioner;
- Ensure that all possible chances of disease spread from the farm is minimized;
- Make sure that information is recorded and compared with data repository;
- Diagnose the disease/ problem primarily based on investigation and initiate steps to contain it;
- Ensure the detailed diagnosis report at the earliest, if sample is sent directly to lab by the farmer;
- Diagnose the disease/ problem precisely and communicate it to the farmer for further;
- Start appropriate treatment (in habitat or Intensive Critical Care Unit) based on diagnosis or instruct for farm isolation, harvest or destroy the stock;
- Use of antibiotic for controlling the disease should be started and only if any bacterial infection is clinically diagnosed;
- Alert the nearby farmers;
- Report to the district level team along with action taken report, once the infection is clinically diagnosed;
- Refer the matter immediately to the district level team for further investigation, if no inference is made by the clinical practitioner;
- Destroy the infected stock safely and ensure proper disinfection of water and farm premises (in case of any contagious disease);
- Must report to the state level committee directly, in case of massive outbreak;
- Maintain all records of the action taken for future reference and data repository;
- Monitor the farm closely for any future outbreak & ensure BMP.

2.2. ACTIVE SURVEILLANCE (IN THE ABSENCE OF DISEASE OUTBREAK)

- Collect sample from farm selected randomly on cash payment;
- Conduct PCR test for the diagnosis of diseases such as NNV/ TiLV/ CEV/ CyHV-2/ EUS for finfish and AHPND/ WSSV/ IHHNV/ HPV/ MBV for shellfish;
- Quantify the load of Aeromonas sp/ Streptococcus sp. for finfish and Vibrio sp for shellfish;
- Conduct residue analysis of chloramphenicol, tetracycline and nitrofuran using ELISA or LC-MS;
- Analyse water for DO, pH, alkalinity, hardness, salinity, nitrate, nitrite, ammonia (ionized and unionized), H2S, iron;
- Analyse feed for the estimation of crude protein, digestible protein and fat;
- Communicate the lab report to the farm along with remedial measures, if any problem is diagnosed;
- Submit monthly report to district level team.

3. LABORATORY LEVEL

- Analyze the sample quickly as per the SoP;
- Furnish diagnosis report to the sender of the sample (farmer/ clinical practitioner), as early as possible;
- Report the case of any disease confirmation to the respective clinical practitioner immediately, if the sample is send by the farmer directly;
- Ensure proper preservation of the sample for future analysis.

4. DISTRICT LEVEL

4.1. RESPONSE FOR REPORTING OF FURTHER DETAILED INVESTIGATION

- Visit the farm within minimum time by the district surveillance team, once the information on disease outbreak is received;
- Examine the initial investigation report and remedial measures undertaken;
- Compare the information and data received with the data repository available;
- Conduct secondary investigation, if required;
- Suggest remedial measures based on the inferences made;
- Refer the matter to the state level committee immediately, if no inference could be made at the district level.

4.2. RESPONSE FOR REPORTING OF INFORMATION ONLY

- Ensure timely diagnosis report from lab & visit the site if possible;
- Closely monitor all the steps undertaken to manage the disease outbreak;
- Alert the farmers of the district through print and electronic media, if it is an epidemic;
- Maintain all records of actions taken for future reference;
- Maintain district level data repository;
- Report to state level with action taken report, if an infection is clinically diagnosed,;
- Report to the Director of Fisheries monthly, about the occurrence of disease in the district;
- Conduct interaction meeting with farmers and other stakeholders once in 6 months;
- Prepare a list of important aquatic animal diseases pertaining to the respective district;
- Keep data on HRD resources available with district along with their status and capabilities.

5. STATE LEVEL

5.1. RESPONSE FOR REPORTING OF FURTHER DETAILED INVESTIGATION

- Convene the state surveillance committee meeting, once the information on disease outbreak is received;
- Visit the farm within the minimum possible time to the site;
- Examine the investigation reports, inferences made and remedial measures already undertaken;
- Conduct a detailed investigation, if required, with the help of KUFOS and ICAR institutes;
- Consult with KUFOS and ICAR institutes for selecting proper preventive measures;
- Suggest remedial measures based on the inferences and consultations made.

5.2. RESPONSE FOR REPORTING OF INFORMATION ONLY

- Ensure proper coordination and cooperation of KUFOS and ICAR institutes;
- Advise the district level team regarding the action to be undertaken;
- Advise on the treatment protocol to be followed;
- Ensure proper documentation and maintenance of state level data repository;
- Maintain all records of the action taken for future reference;
- Alert the farmers in the state through media, if a serious outbreak has happened;
- Report quarterly to GoI, about the occurrence of aquatic animal health disease (OIE listed disease should be separately noted).

Details of OIE listed pathogens (2020)

Fish

Epizootic haematopoietic necrosis virus
Infectious salmon anaemia virus
Salmonid alpha virus
Infectious haematopoietic necrosis virus
Koi herpes virus
Red sea bream irido virus
Spring viraemia of carp virus
Viral hemorrhagic septicaemia virus
Aphanomyces invadans
Gyrodactylus salaris

Crustacean

Acute hepatopancreatic necrosis disease
Hepatobacter penaei- Necrotising hepatopancreatitis
IHHNV
IMNV
Macrobrachium rosenbergii nodavirus
TSV
WSSV
YHV
Aphanomyces astaci

Mollusc

Perkinsus olseni
Abalone herpesvirus
Bonamia astreae
Marteilia refringens
Bonamia exitiosa
Xenohaliotus californiensis

III. DISEASE INVESTIGATION

Mortality pattern in a culture system can give an indication of the possible causes of the disease. For any kind of relatively uniform occurrence of mortality, irrespective of cultured species, suspect for fluctuations in environmental parameters. On the other hand, for sporadic or random mortality pattern, the aquatic animal from affected stock needs to be sent immediately to diagnostic facility. Any mortality of more than 0.3% per day should be investigated. For the cases where spread of mortality is observed, the affected stock has to be isolated to prevent further spreading. Mortality of more than 1.5% per day should be treated as an epizootic. In clinical cases of disease (mortality >0.5% per day), 10 moribund fish or shellfish are generally sufficient for a sample. If no excessive mortality or clinical disease is apparent, a larger sample size of 60 animals may be required. Depending upon individual circumstances, sample size may vary between 10 and 60. Samples should be collected from feed trays, sides and middle of the pond using cast net.

1. ANAMNESIS (Case history/ records)

Prompt, accurate and complete record keeping about the aquatic animal is imperative for good health management and should include the following:

- ✓ Farm :- Name, address, GPS co-ordinates, phone number, e-mail, registration number;
- ✓ Nature of stock :- Fish or shrimp;
- ✓ Purpose of rearing:- Food, ornamental or seed production;
- ✓ Water:- Source, quality parameters, bloom, colour, odour;
- ✓ Farming system:- Pond, tank, Re-circulatory Aquaculture System (RAS), Cage, Polyculture, Integrated farming, flood plains;
- ✓ Nature of water body:- Open or closed;
- ✓ Animal:- Species;
- ✓ Seed:- Source, size, stocking density, screening conducted;
- ✓ Feed:- Nutritional labeling, type, size, rate, regime, storage condition;
- ✓ Growth performance:- Days of Culture (DoC), average size, anorectic;
- ✓ Handling:- Grading, sampling, cleaning(cages), water exchange, partial harvesting, introduction of new animal;
- ✓ Recent changes in husbandry practices, if any;
- ✓ Recent pathologies and treatments (The more precise and consistent the farm records are the more likely the pathologist reaches a sound diagnosis).

2. ONSITE OBSERVATION OF HABITAT

- ✓ Water:- Temperature, pH, DO, ammonia (ionized and unionized), hydrogen sulphide, alkalinity, salinity, hardness, transparency, nitrite, nitrate, phosphate;
- ✓ Soil:- Colour, silt content, pH, Eh, hydrogen sulphide;
- ✓ Plankton:- Bloom, colour;
- ✓ Macrovegetation:- Weeds, filamentous algae if any;
- ✓ Reactions to stimuli in its environment:- Attraction towards feed, response to external stimuli;
- ✓ Behavioural abnormalities: -

  For fish, observe for flashing, circling, gasping, surfacing, whirling, congregation near inlet/ water surface, vertical swimming, vertical hanging, dorsal/ lateral recumbency, rubbing, lethargic movement, isolation, listlessness, anorexia;

  For shrimp, observe for surfacing, congregation near inlet/ water surface, vertical swimming, lethargic movement, coming to the sides of pond during day time, isolation, listlessness, cannibalism, anorexia;
- ✓ Stunted/ differential growth;
- ✓ Presence of dead and moribund animals:- Number/ percentage per day and cumulative;
- ✓ Congregation of predatory birds near the pond premises;
- ✓ Faeces floating on water surface.

3. ONSITE EXAMINATION OF FEED

- ✓ Smell;
- ✓ Colour changes if any;
- ✓ Presence of fungus on physical examination;
- ✓ Water stability;
- ✓ Presence of feed crumbs.

4. ON SITE PHYSICAL EXAMINATION OF AQUATIC ANIMAL

4.1. Fish

- ✓ Physical measurement:- Length, weight;
- ✓ Body:- Observe with magnifying glass for discolouration, reddening, haemorrhage, ulcer, wound, abdominal swelling/ dropsy, emaciation, tumour, deformity, presence of parasite/ epizoic growth, nodules;

- ✓ Eye:- Exophthalmia, enophthalmia, cloudy eye, haemorrhage;
- ✓ Fin:- Erosion/ rot, ragged/ torn, haemorrhage at the base, presence of parasite/ epizoic growth;
- ✓ Scale:- Protrusion, loss;
- ✓ Vent:- Reddening, swelling, petechiae (pin point haemorrhage), faecal trailing;
- ✓ Mucus:- Increased production, lack of production, colour change;
- ✓ Gill:- Observe both sides for change in colour (pale/ red/ orange/ yellow/ black), rot, necrosis, gas super saturation, nodules, presence of parasite/ epizoic growth;
- ✓ Buccal cavity:- Haemorrhage, presence of parasite/ epizoic growth.

4.2. Shrimp

- ✓ Physical measurement:- Length, weight;
- ✓ Exoskeleton:- Discolouration, presence of spots (white/ black/ brown), loose shell, wound, deformities, presence of parasite/ epizoic growth, dull appearance (loss of sheen);
- ✓ Rostrum:- Broken, bent/ deformation;
- ✓ Antenna:- Cut, loss;
- ✓ Appendage:- Reddening, broken, loss, necrosis;
- ✓ Telson:- Erosion/ rot, presence of parasite/ epizoic growth;
- ✓ Eye:- Cloudy;
- ✓ Gill:- Change in colour (pale/ red/ orange/ yellow/ black), rot, clogging, necrosis, branchiostegal blister, presence of parasite/ epizoic growth;
- ✓ Muscle:- Flaccid, discolouration (pale, opaque, whitish, reddish, yellowish, bluish);
- ✓ Gut:- Empty, colour change (white);
- ✓ Hepatopancreas:- Swollen/ shrunken, colour change (yellow/ brown/ pale).

5. SAMPLE COLLECTION & TRANSPORTATION

5.1. Aquatic animal

- ✓ Collect the specimen as early as possible after the onset of clinical symptoms and preferably before the adoption of therapeutic practice;
- ✓ Moribund/ live animal exhibiting clinical signs are preferred for diagnosis;
- ✓ Do not collect live sample which are feeding aggressively;
- ✓ Take physical measurements immediately after collection;
- ✓ While sampling, affected animal should be noted for the colour, mucus, clear eyes, and red/ pink gills;

- ✓ Number of samples to be taken is based on prevalence of disease and the details are given in the table below:

Lot size	No. of sample to be taken based on prevalence (if the prevalence is not known, then it is assumed to be 10%)						
	0.5%	**1.0%**	**2.0%**	**3.0%**	**4.0%**	**5.0%**	**10%**
50	46	46	46	37	37	29	20
100	93	93	76	61	50	43	23
250	192	156	110	75	62	49	25
500	314	223	127	88	67	54	26
1000	448	256	136	92	69	55	27
2500	512	279	142	95	71	56	27
5000	562	288	145	96	72	57	27
10000	579	292	146	96	72	57	27
100000	594	296	147	97	72	57	27
1000000	596	297	147	97	72	57	27
>1000000	600	300	150	100	75	60	30

(Source : FAO)

- ✓ For molecular diagnosis alone, 5-6 moribund samples are sufficient if the clinical signs are obvious; otherwise randomly collect 60 samples from different points and make into 12 pools of 5 numbers each. In the case of larval stages, collect 200 samples from different points of the tank, randomly group into 5 pools of 30 numbers each and lively transport to the laboratory in oxygen packs. Larvae fixed in 70% ethanol are also suitable.
- ✓ Pack immediately the collected live/ moribund animal as sample, after removal from water, in double plastic bags with a minimum amount of water (not more than one-third full) from the same holding facility (Do not use chlorinated water) and fill the remaining space with oxygen, if possible.

  Note: The bags should be sealed tightly with rubber bands/rings and packed inside a strong box, preferably lined with styrofoam provided with a small amount of crushed ice kept in a separate sealed bag so that it is packed around the specimen just enough to keep the water cool. This box is then taped securely and may be packaged inside a cardboard carton. Seal the box securely and mark the following: 1. Scientific Specimens; 2. Perishable; 3. Notify Upon Arrival (Provide Phone number of the addressee).
- ✓ In case live specimen is not available, the tissue from the recently dead animal should be dissected out, preserved in ice and labeled;

Note: Care should be taken to prevent direct contact of ice with the specimen. Adequate amount of ice must be layered with proper sealing to prevent leakage. Wet ice is preferred for materials intended for bacteriological examination. Samples for microbiology should be processed within 24 hours. Do not preserve sample in alcohol or formalin for culture based diagnosis; which will destroy parasites, virus and bacteria. For virological analysis, the samples must be homogenised and inoculated in cell lines immediately.

- ✓ A good clinical history of the case should always accompany the specimen;
- ✓ Samples are transported to the laboratory in wide mouthed corked glass containers under insulated condition (Cotton plugged tubes are unacceptable). All containers must be labelled. It is also advantageous, if the inner container is carefully wrapped in absorbent paper or other such material to absorb shock and any fluid that may be spilt;
- ✓ Sample should reach the laboratory not later than 4 hours after collection and the transportation period can be extended up to 24 hours, if the material is well packed and kept at 2^0 to 4^0C.

5.2. Water

- ✓ Collect water sample from 1 metre depth (composite mixture of all four sides and middle);
- ✓ Water samples should be held in ice and transported in sealed and insulated containers and two-100ml water samples can be collected from the opposite ends of the pond.
- ✓ The temperature and dissolved oxygen of the water sample is determined in-situ because it will change during transport;
- ✓ An additional set of DO bottles is used for water collection to determine DO, BOD and COD through titration;
- ✓ If TVC is above 5 log CFU/ ml, collect water sample for estimating total count of *Aeromonas* and *Vibrios.*

5.3. Soil

- ✓ Collect top soil from all four sides and middle and make into a composite mixture to determine NPK ratio, C:N ratio and Iron.

5.4. Feed

- ✓ Collect feed sample from the bag which was opened last (used for feeding at the time of mortality).

5.5. Faecal matter

- ✓ Collect samples of faecal matter or aquarium debris, concentrate by floatation with standard sodium nitrate (Specific gravity-1.18 to 1.20) and observe the wet mount under low power microscopy for any parasitic infestation. It is the least stressful observation procedure, however considered as the least sensitive procedure. (optional)

6. CLINICAL EXAMINATION AND NECROPSY

6. 1. Fish

- ✓ Note the behavioural pattern of the affected fish;
- ✓ Collect blood sample for bacteriology (inoculate into suitable media: brain heart infusion agar and TSA for freshwater fish and Zobell marine agar, TCBS agar and Tryptose yeast extract salt agar and 5% foetal bovine serum for marine fish and check for any bacterial growth), serology (conduct relevant tests for health assessment and pathogen screening), blood smear preparation (Giemsa/ Wright stain) and examination;
- ✓ Examination of mucous smears from gill and skin;
- ✓ Euthanize fish following standard procedure, if it is alive (sever the spinal cord by a sharp incision behind the brain, or pithing the brain by a sharp object);
- ✓ Examine for external lesions;
- ✓ Collect swab from external lesion/ sore and inoculate aseptically into suitable media (Brain heart infusion agar and TSA for freshwater fish and Zobell marine agar, TCBS agar & Tryptose yeast extract salt agar with 5% foetal bovine serum agar for marine fish);
- ✓ Examine the skin and fin wet mounts under the microscope for the presence of parasites;
- ✓ Remove operculum and examine gill and its wet mount under the microscope for the presence of parasites;
- ✓ Aseptically disinfect the outer layer of fish by flooding with 70% alcohol;
- ✓ Make a cut along the ventral surface immediately anterior to the anus for collection of samples from internal tissues;
  i. Insert scissors and cut forward to the base of the pectoral fins, then through the pectoral girdle and then cut up the edge of the operculum to the top of the abdominal cavity and then back towards the vent (make sure that incision is slightly above the intestinal tract so that the internal organs are not ruptured

because it may result in contamination of target tissue) so that the flap of the body can be removed to expose the internal organs;

ii. Pull intestinal viscera and air bladder using butt end of sterile inoculating loop or forceps for exposing the kidney;

iii. Collect a piece of kidney tissue using sterile forceps and streak directly on to agar plate;

- ✓ Note down fluid accumulation if any, in abdominal cavity and its colour;
- ✓ Check for the presence of any parasite in the viscera;
- ✓ Observe for peritonitis if any;
- ✓ Observe the internal organs (kidney, liver, spleen and heart) and note down the colour, consistency, size, shape;
- ✓ Examine various internal organs for the presence of any parasites;
- ✓ Cut into the cranium, posterior to the eyes to examine the brain. When the cavity is exposed, lift the brain out with forceps by placing them under the entire brain and pulling in an upwards motion;
- ✓ Collect samples by following standard procedures for bacteriological, virological, molecular, parasitological, mycological and histopathological investigations;
- ✓ Dispose-off the sample by incineration or disinfection and burial.

6. 2. Shrimp

- ✓ Note the behavioural pattern of the affected shrimp;
- ✓ Examination of cuticle and smear from gills;
- ✓ Anaesthetise shrimp by cold shock;
- ✓ Examine for external lesion;
- ✓ Collect haemolymph from the cephalothorax region immediately by pericardial puncture or from the ventral sinus using a sterile tuberculin syringe, prepare smear by placing a drop of haemolymph for observation and inoculate aseptically with a sterile loop into suitable media: Zobell marine agar and TCBS agar for bacteria, Mycological agar for fungi (Another method for collecting haemolymph is by cutting antennae after disinfecting with alcohol and by streaking the haemolymph that comes out on agar plate with a sterile loop);
- ✓ Collect swab from external lesion and affected tissues/ infected larvae and inoculate aseptically into suitable media as above;
- ✓ Examine gill and its wet mount under the microscope for the presence of parasites;
- ✓ Aseptically disinfect the outer layer of shrimp by flooding with 70% alcohol;
- ✓ Slit the cuticle with dissecting scissors along the side of the body from the sixth abdominal segment to the cuticle overlying the "head region" (cephalothorax). From

there, angle the cut forward and upward until it reaches the base of the rostrum. Avoid cutting too deeply into the underlying tissue. Shrimp over 12 g should be transversely dissected, at least once, posterior of the abdomen/ cephalothorax junction and again mid-abdominally;

- ✓ Observe the internal organs (hepatopancreas, gut, heart, lymphoid organs) and note down the colour, consistency, size & shape;
- ✓ Prepare squash of hepatopancreas and stain with Giemsa for viral occlusions and inclusion bodies ;
- ✓ Collect samples of hepatopancreas, gut, heart, lymphoid organs, gills, muscles & pleopods aseptically by following standard procedures and cut surface of organ is streaked on agar plate medium such as Trypticase Soy Agar (TSA) with 2% NaCl for bacteriological investigations;
- ✓ In the case of smaller live samples like larval stages, the whole animal is crushed after rinsing in sterile sea water or 2.5% NaCl and the exudate is streaked onto agar medium plate for incubation (smaller animals should be transported in ice pack in sterile vials with sterile sea water);
- ✓ Dispose-off the sample by incineration or disinfection and burial.

Note:

a. *Live specimens have to be handled aseptically in a short period;*
b. *Use sterile snips/ scissors and forceps for each specimen to prevent cross contamination;*
c. *Used snips/ scissors and forceps should be decontaminated;*
d. *Bacteriological samples should be taken first to avoid contamination;*
e. *For parasitic observation, the wet mount should be observed immediately because most of the parasites die on removal from the host;*
f. *For histopathology live/ moribund specimen should only be used. (For parasitic examination immediately dead and brought in iced condition to the lab may be used)*

7. PREPARATION OF SAMPLE & DETAILED INVESTIGATION AT LAB

7.1. Molecular diagnosis (Virological)

- ✓ Tissue samples from organs, preferably of kidney or spleen, are mostly preferred for virological analysis in fish, and the details are given in the table below:

Fish size in length	Tissues
≤2.5 cm	Use whole fish
2.5 to 4.0 cm	Cut off and discard head and tail
4 to 7 cm	Use viscera
≥7.0 cm	Kidney, spleen, heart, brain and/ or suitable tissue appropriate for specific pathogen being tested.
Adult fish	Kidney, spleen, ovarian fluid (female)/ seminal fluid (male) and / or suitable tissue appropriate for specific pathogen being tested

- ✓ In the case of shrimp, tissue samples from pleopods, muscles, gills, gut and hepatopancreas are collected by cutting out using sterile scissors;
- ✓ In the case of larval stages of shrimp, whole body is used after rinsing in sterile sea water or 2.5% NaCl and removing eyes, as it has PCR inhibiting substances (Sub sample of 30 larvae should be taken aseptically for DNA extraction);
- ✓ If the sample is intended to be send to any other disease diagnostic laboratory, the collected sample is put in tightly capped container with fixative (80-95% ethanol) and should be kept in dry ice or liquid nitrogen;
- ✓ Dissect out 0.5 g tissue from each collected sample and transfer into two collection vials;
- ✓ Add cold Hank's Balanced Salt Solution (HBSS) to one of vial, so that the sample is covered;
- ✓ Even though individual samples are preferred; samples may be pooled, if necessary, but should not be more than 5 individuals into a single sample and samples from individuals must be approximately the same size;
- ✓ For blood/ mucus/ faecal samples, add appropriate amount of antibacterial/ antifungal agent and transport at cool conditions;
- ✓ Note: Suitable antibiotics are Gentamycin (1000 µg/ml) or Penicillin (800 IU/ml) and streptomycin (800 µg/ml). Antifungal compounds, such as Mycostatin or Fungizone, may also be incorporated into the transport medium at a final concentration of 400 IU/ml. Serum or albumin (5–10%) may be added to stabilise the virus if the transport time exceeds 12 hours.
- ✓ Samples should be kept at 5-18°C (do not freeze) during shipment.
- ✓ Note: Sample is maintained at 0-4°C up to 48 hours transportation. Otherwise the sample may be destroyed by drying or bacterial decomposition;
- ✓ Conduct molecular diagnostic analysis as per standard procedures.

7.2. Microbiological analysis

- ✓ Sample for microbiology analysis is taken during clinical examination and necropsy;
- ✓ Conduct microbiology analysis as per standard procedures.

7.3. Histological analysis

- ✓ Live (after anesthetizing)/ moribund fish should only be used. Never freeze tissue meant for histology;
- ✓ Tissue samples should be fixed with fixative in a wide mouth plastic bottle as early as possible after removal from water or time of death prior to transporting to the lab, since, fish rapidly autolyze;
- ✓ In the case of larger fish (>6 cm), dissect-out tissue having preferable size of <1 cm from specified organ with sharp blade or scissors and transported in fixative;
- ✓ In the case of small fish, the specimen is slit open/ inject the fixative into the intestinal cavity before immersing in fixative to ensure better penetration of fixative;
- ✓ If the specimen is a larger shrimp (>12g), inject the fixative at 3 or 4 places (cephalothorax, anterior abdomen and posterior abdomen) and put in the fixative for 48 hours;
- ✓ (Note: The cuticle should be slit open on both sides of the shrimp from the sixth abdominal segment to the rostrum using scissors, before placing in fixative to ensure the proper penetration of fixative. Organs are dissected out and fixed separately. Hepatopancreas should be immediately fixed post death.)
- ✓ In case of post larvae (PL) and small shrimp (<12 g), directly immerse the specimen in fixative for 24 hours;
- ✓ The volume of fixative must be at least 10 times the volume of the tissue;
- ✓ The fixative must be replaced after 24hours;
- ✓ The commonly used fixatives are 10% neutral buffered formalin (NBF) for fish and Davidson's fixative for shellfish;
- ✓ Formalin based fixatives are hazardous, so skin and eye contact/ inhalation should be avoided;
- ✓ Fixatives in alcohol should be specifically noted on the container during transport due to their inflammable nature;
- ✓ Seal the container properly;
- ✓ Details of sample should be labelled on paper and placed inside the fixative container (labelling should be made with pencil, as alcohol and fixatives tend to wash off pen marks on the container);
- ✓ Samples put in Bouin's fluid or Davidson's fixative should be transferred to 70% alcohol after 24-48 hours;
- ✓ Conduct histological analysis as per standard procedures.

7.4. Parasitological analysis

- ✓ Preferably live or moribund samples are required for parasitological examination and handle the animal as gentle as possible;
- ✓ If the animal is dead, bring the samples (or faecal material) in normal saline under refrigerated/ iced condition;
- ✓ Sample should be freshly killed, without anaesthetic, by cutting through cranium or through spinal cord;
- ✓ Animal should be kept moist throughout the examination;
- ✓ Parasites are more easily recognised and identified when live or immediately after death, once the animal is dead, ectoparasites may leave the host;
- ✓ Examine the entire body under low power using a stereo microscope.
- ✓ Examine under the pectoral fins/ appendages using microscope;
- ✓ Collect mucous by scrapping with a scalpel in an 'anterior to posterior' direction and the collected mucous and epithelial cells should be placed on a slide in a drop of water and examined under the microscope for the presence of any parasites;
- ✓ Collect blood via caudal vein/ artery or heart and thin blood film should be prepared on a slide. The film should be air dried and fixed in absolute methanol for 10 min and stained using an appropriate stain. The prepared blood smear should be examined for the presence of any parasites in the blood;
- ✓ Put a sterile swab along the surface of an external lesion and the material from the swab should be transferred to normal saline or PBS by pressing or rolling the swab against the interior of the tube and examined directly under microscope for the presence of any parasite in the external lesion, (Kept cold if there is any delay for examination);
- ✓ Remove gill filament/ tissue from anesthetized fish and examine the tissue directly under microscope for parasites or kept cold preserved for further examination;
- ✓ Carry out detailed microscopic examination of peritoneal cavity and all internal organs;
- ✓ Remove the gut, cut open and directly examine under microscope;
- ✓ Impression smear preparations from heart, liver, spleen, kidney and gonads are made and observed under microscope;
- ✓ Smear preparations of contents from gall bladder and urinary bladder are made and observed under microscope;
- ✓ In the case of fish, remove skin and slice the muscle and examine for helminth cysts/ larvae and protozoan cysts.

7.5. Biopsy/ Non-lethal sample (fish)

(i) Fin clips:

- ✓ Spread the fin using finger and aseptically cut a small piece of fin tissue using scissors;
- ✓ Place tissue in labelled screw cap tube;
- ✓ Rinse scissors and forceps in 70% ethanol after each sampling to minimize contamination risk.

(ii) Gills:

- ✓ Open gill chamber with the aid of a sterile scissor;
- ✓ The tip of several primary lamellae is cut to prevent bleeding and transferred to a slide on which cover slip is placed without trapping air bubbles;
- ✓ After gross examination, observe under low magnification microscopy;
- ✓ Take sample of mucous by gently inserting cotton buds under the operculum and rolling it over filaments;
- ✓ Spread the cotton buds in a slide and observe under low magnification microscopy;
- ✓ Rinse scissors and forceps in 70% ethanol after each sampling to minimize contamination risk.

(iii) Skin:

- ✓ Take skin scraping and observe for the presence of any pathogen or other abnormalities;
- ✓ Perform skin scrapings by a scalpel, spatula or covers lips from cranial to caudal direction. For this, the fish should be held with moist towels and damages/ drying should be avoided while scraping. The leading edge of lesion is the preferable area for sampling, as this area usually harbour the pathogens;
- ✓ Immediately transfer the scrapings to a glass slide, carrying a drop of water (same holding water) and observe under low magnification microscope after putting the cover slip.

(iv) Scales:

- ✓ Scrape approximately 5-10 scales from the side with a sterile pocket knife or scalpel and observe under the microscope.

(v) Collection of Blood:

- ✓ Collect blood from the caudal vein by inserting the needle into the musculature perpendicular to the ventral midline of the caudal peduncle of the fish until it reaches the spine or blood enters the syringe. The vein is ventral to the overlying

spine. This blood vessel can also be sampled laterally by inserting needle just below the lateral line at 45° angle to the long axis of the fish in a cranial direction;
- ✓ Withdraw the needle slightly, once contact with the spine is made;
- ✓ Carefully remove the needle from the syringe before transferring blood into the vial.

8. DISEASE DIAGNOSIS BASED ON PATHOLOGICAL SIGNS

8.1. Fish

Clinical Signs of Fish	Probable Causes
Skin:	
Red areas on the body surface	External parasites (*Ichthyophthirius/ Trichodina/ Oodinium/ Dactylogyrus*)
	Internal infection, abdominal dropsy
	Environmental damages (acid disease, caustic effect of chemicals etc)
	Due to handling
Excess mucous production	Ectoparasitic infection, environmental damages/ chemicals
Ulcerations	Handling methods (eg: netting), ectoparasites, bacteria
Cotton wool like white grey coating which collapse in air	Fungus (eg: *Saprolegnia*)
Velvet- like bluish white turbidity	Infestation with parasites (*Costia*, *Trichodina*, *Chlidonella*, *Gyrodactylus*, etc) Effect of chemical (Alkali)
Pearl to raspberry like growths	Lymphocystis
Long to short oval nodules originating deeper down, grey–white to whitish, black spots	Metacercarial cysts
Black colouration of whole sections of the body, usually starting posteriorly	Whirling disease, Viral hemorrhagic septicemia of trout

Blisters	Abdominal dropsy of cyprinids, bacterial kidney disease, furunculosis, ulcer disease
Lepidorthosis (Raised Scales)	Inflammation and swelling of the skin due to diseases caused by bacteria
Redness and prolapse of the anus, often exuding yellowish mucous when squeezed	Inflammation of intestine caused by viral or bacterial Infection Environmental irritants, unsuitable food irritating intestine, intestinal parasites (Eg: *Eimeria*), Furunculosis
Sunken eyes	KHV, severe internal illness, metabolic disturbances, starvation, chlorine poisoning
Opaqueness of cornea or eye	Infection due to *Streptococcus* sp., parasites (Metacercariae) or chemicals
Raised operculum	Tumour of thyroid gland or gill swelling
Frayed fins	Bacterial infection, metabolic disturbances, obstructed circulation due to internal infection
Discolouration in line or patch form	Muscular damage due to parasites (Micro/ Myxosporidia), Fungi (*Aphanomyces*) etc
Exophthalmos	Swelling behind the eye ball caused by infectious diseases, parasites or rarely of genetic origin (telescope fish)
Gills:	
Pallor	Anaemia, oxygen deficiency
Dark colouration of abnormal intensity	Circulatory obstruction due to pathogens or chemicals
Copious mucus secretion	Chemical irritant in water or due to parasites
Gill extremities destroyed, frayed, cauterised	Chemical irritants in the water or more rarely due to parasites

Marked swelling of individual gill filaments	Bacteria, avitaminosis, irritation by parasites (*Ichthyophthirius*, *Dactylogyrus*)
Yellowish nodules of pinhead size	Myxosporean cysts
Small white round spots	*Ichthyophthirius*, *Cryptocaryon*
Longish white spots	Gill crustaceans (*Ergasilus*) or *Dermocystidium* cysts
Cotton wool like coating on the gills, collapses in air	*Saprolegnia*
Brownish colouration, often at gill extremities	Gill Rot (*Branchiomyces*)
Whitish turbidity	*Costia*, *Chilodonella*, *Trichodina*
Bluish white, velvety coating of gills	*Oodinium*
Small worms, colourless to yellowish	Monogenetic Trematodes
Nodules	Tumour
Bubbles	Supersaturation of water
Gall Bladder:	
Presence of very motile, microscopically small unicellular organisms	*Hexamita* (Protozoan)
Presence of non-motile spores	Myxozoa, Microsporidia
Liver:	
Pale to yellowish discolouration	Viral hemorrhagic septicaemia (VHS), Lipoidosis, Infectious dropsy, Furunculosis, Bacterial Kidney Disease (BKD), Infectious Pancreatic Necrosis (IPN)
Patchy inflammation of liver, brownish spots	VHS, Necrosis, Cirrhosis, Furunculosis
Pin head to Pea-sized ball like cysts with worms	Cysts with plerocercoids of *Triaenophorus*, Nematode cysts
Yellowish white cysts, slightly large, without worms	Hepatoma
Whitish, minute cysts	*Ichthyosporidium*, Piscine tuberculosis

Yellowish, minute ball like cysts	Metacercaria
Round, long worms	Nematodes
Gut:	
Redness and inflammation of alimentary canal	Infections, parasites, poisoning, faulty feeding
Distension of stomach or parts of intestine	Disturbances of digestion, inflammation of intestine
Small yellowish nodules on the outer surface of the intestine	Anterior parts of acanthocephalans pushed through the intestinal walls
Swelling of pyloric caeca	Parasites, (usually cestodes)
Excessive mucus secretion of intestine	Intestinal irritation
Small, yellowish white nodules on interior surface of intestine	*Eimeria,* Myxosporeans
Round worms, white or yellow, adhering	Acanthocephalan (Thorny headed worm)
Long, white flat worms, segmented	Cestodes
Whitish, flat, unsegmented worms	Usually plerocercoid larvae of cestodes
Pale white, yellowish, reddish or brownish worms, flat, unsegmented, usually very small	Trematodes (flukes)
Small unicellular parasites in intestinal contents	*Hexamita* (Protozoa)
Eggs within intestinal contents	Usually eggs of worms
Kidney:	
Bloody inflammation of kidney, swelling	Viral Hemorrhagic Septicemia of Trout, Bacterial Kidney Disease
Cyst like swelling of kidney	Renal cysts
Kidney containing encysted helminth eggs	*Sanguinicola* (blood fluke)
White nodules in kidney	Visceral granuloma, *Ichthyosporidium*, Bacterial Kidney Disease
Head kidney abnormally thick, with grey colouration	*Sphaerospora*

Granulomas in kidney	Bacterial kidney disease
Loose, crunchy, white coloured deposits of Ca_3PO_4 resulting in the dilation of tubules/ ducts and thickening of glomerulus (Nephrocalcinosis)	CO_2 in water more than 12 ppm
Necrosis and mats of fungus in the initial stages developing into granulomas with few hyphae. Presence of giant cells fusion of macrophages in kidney	*Exophila* infection
Pancreas:	
Pancreas necrotic, muscle degeneration	IPN
Necrosis and haemorrhages	IHN
Lipid tissue associated with exocrine part (acinar cells)	Vitamin E deficiency or rancidity of feed
Spleen:	
Notably enlarged	Presence or consequence of an infection
Small white nodules on spleen	*Ichythosporidium*, Bacterial Kidney Disease, Tuberculosis
Gonads:	
Inflammation of gonads	Furunculosis, VHS, *Ichthyosporidium*, Abdominal dropsy of cyprinids, Poisoning
Cyst in ovary, necrosis/ fibrosis leading to sterility	*Pleistophora ovariae*
Round, coiled worms in between parts of gonads	Nematodes
Swim bladder:	
Loss of balance due to loss of control of gaseous secretion	Unknown, nutritional factors or excess dusty feed suspected
Chronic inflammation with necrosis and excess exudates in gas bladder	Unknown, Virus suspected
Oedema of walls of gas bladder	Chronic bacterial infections or other systemic infection

Parasites (Nematodes) and Cyst (*Eimeria*) in the lumen	
Heart:	
Yellowish- white ball like nodules in the heart	Metacercariae, Myxosporeans *Ichthyosporidium* (fungi), Tuberculosis
Worm eggs in heart blood	*Sanguinicola*
Lesions in heart muscle and pericarditis	Bacterial (BKD)
Cardiac oedema	Osmoregulatory failure Direct cardiac failure with renal Branchial circulatory failure
Haemorrhages	Virus
Resting spores and fungal hyphae in cardiac muscle resulting in cardiac failure	*Ichthyophonus*
Muscles:	
Muscles containing microscopic ball like cyst with spores	Microsporidia
Cyst formation, very often pigmented and mild fibrosis	Metacercaria
Muscles containing flat, unsegmented whitish worms	Cestode larvae
Parasites with major portion of head buried inside muscle	Anchor worm
Resting spores and fungal hyphae, chronic granuloma formation	*Ichthyohonus*
Liquefactive necrosis	Muscle parasites like microsporidians/ Myxosporeans
Ulcerations in muscle	Infectious abdominal dropsy, furunculosis
Small, white to grey-brown nodules	*Ichthyosporidium* (fungus)

8.2. Shrimp

Clinical Signs of Shrimp	Probable Causes
Body colour or markings:	
Reddening of the legs and body	Gill associated virus (GAV) related disease, Vibriosis
Black marks or lesions	Healed wound, Bacterial shell disease Black splint disease
White spots in the cuticle	Non-viral conditions, Exotic viral disease, White Spot Syndrome Virus (WSSV)
White muscle	White cotton disease, Extreme pond temperatures (heat stress)
Red midgut	Haemocytic enteritis (gut infection)
Gill colour:	
Red gills	Stress
Black gills	Significant organic fouling in the pond bottom or algae die-off Blue-green algae growing on gill filaments Infectious damage to the filaments and melanisation Exposure to iron salts
Deformities or external problems:	
Tail cramping	High temperatures and/or salinities
Runts in the crop	Disease (haemocytic enteritis) or genetic growth variation
Tumours or abnormal growth(s) on the body	Genetic disease, chemical pollution
Behaviour:	
Abnormal swimming, burrowing, congregating	Temperatures (too cool or too hot), heat stress, low dissolved oxygen, sudden drop in pH, high salinity, thick algal bloom, shell or gill fouling
Prawns fail to moult	
Empty gut:	
Anorexia	Stress, rancid or poor-quality feed, lack of feed, poor water quality

9. HEALTH ASSESSMENT OF AQUATIC ANIMAL

9.1. Physiological Test:

Name of test	Activity	Sign of healthy seed
Feeding test	Place with preferred food	Should have full guts in 10–15 minutes
Swirl test	Swirled in water	Should realign and start swimming into the current
Salinity stress test	Expose to a salinity variation of 5-10 ppt for 2 hours	Should survive and resume feeding within 24 hours of the test
Temperature stress test	Keep with a temperature variation of 6-8^{0}c for 5-10 minutes	Should survive and recover quickly when put back in ambient water temperature.
Formalin stress test	Place in water having 100 ppm formalin with aeration	Should survive more than 80%
Size test	Measure the size	Should have uniform size

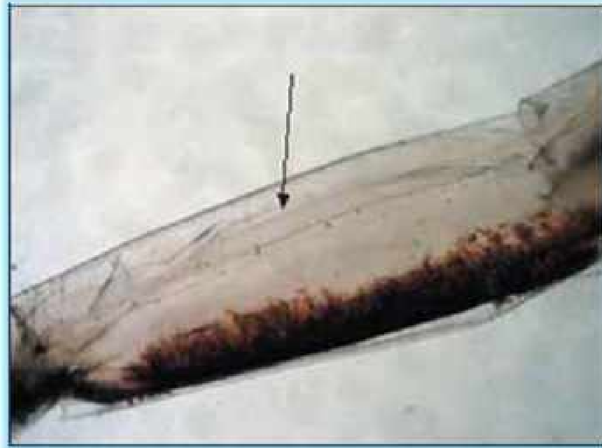
Note: Moulted one is less physiologically tolerant and is more susceptible to stress. Hence, assessments of larval quality can never be standardised during moult stage of shrimp.

9.2. Visual Observation of PL:

Criteria	Healthy seed	Unhealthy seed
Colour	Light grey/ black/ brown or transparent	Reddish/ pinkish blue reflects stressed post larvae.
Activity	Swim actively, does not clump together and remain evenly distributed. In a basin they may not move always but jump when mildly tapped on the container. When water current is created, the larvae try to move against the current.	They tend to settle in clumps. React feebly to gentle tap on container.
Feeding	Readily accepts and eat feed.	Reluctant to accept feed.

9.3. Microscopic Observation of PL:

Criteria	Healthy seed	Unhealthy seed
Shell	Clean shell is a sign of regular moulting frequency and good health	Dirty shell or necrosis (wounds) on the shell or presence of protozoan.
Muscle	Clear, smooth and thick muscle completely fill the space between gut and the underside	Grainy and thin whitish or opaque muscle not filling the space below gut
Gut	Full gut with food	Empty or incompletely filled gut
Muscle to gut ratio	Tail muscle to hind gut ratio 4:1 or more	Ratio less than 4:1
Pigmentation	Chromatophores well defined and located along the mid ventral line	Chromatophores diffused or expanded
Appendages	Intact, without any deformity or having bent setae uropods remaining spread like a fan.	Broken or deformed appendages.



Postlarva tail section with empty gut visible

Postlarva last tail segment with gut visible

Postlarva head and rostrum

Postlarva with broken rostrum
(Photos courtesy : Australian Prawn Farming Manual)

IV. COMMONLY OCCURRING AND EMERGING FISH DISEASES

1. VIRAL DISEASES

1.1. Koi Herpes Virus Disease (KHVD)

Causative agent: Cyprinid herpesvirus-3 (CyHV-3)/ Koi herpesvirus (KHV)

Host: Koi carp and common carp. All age groups from juveniles upwards, appear to be susceptible to KHVD. Outbreaks are more severe and fish is most susceptible at 18-28°C. Gills are the major portal of viral entry.

Clinical signs: Discoloration of the gills from red to pale with white necrotic patches. Enophthalmia, pale patches on the skin, increased mucus production on skin and gills. The primary gill infection is followed by secondary parasitic infection.

Transmission: Mainly horizontal. Vertical transmission (egg associated) also reported. Fish, parasitic invertebrate, piscivorous bird and mammals can act as vectors.

1.2. Goldfish Herpes Virus Disease

Causative agent: Cyprinid herpesvirus-2 (CyHV-2)/ Herpes viral haematopoietic necrosis (HVHN)

Host: Goldfish

Clinical signs: Large scale haemorrhages on the body, fins and gills, anaemia, lepidorthosis, necrotised gills, protruded anus and shrunken eyes. White nodular necrotic foci on spleen and kidney, along with fusion of gill lamellae are seen. Fading of pigments leading to a whitish appearance of skin and scales is also observed.

Transmission: Horizontal. Low temperature is a predisposing factor.

1.3. Infectious Spleen and Kidney Necrosis Virus (ISKNV)

Causative agent: ISKNV-like viruses a group of viral agents in the genus Megalocytivirus, family Iridoviridae

Host: Freshwater fish (particularly cichlids, gouramis and poeciliids), brackishwater and marine fish

Clinical signs: Mortality is 50 - 100%. Lethargy, anorexia, respiratory distress, changes in body colour, exophthalmia and abdominal distension (due to ascites or enlargement of organs) are the clinical signs observed during the disease.

Transmission: Horizontal. There is evidence that some species may be long-term asymptomatic carriers of ISKNV-like viruses, and prevalence in infected populations may be high.

1.4. Carp Edema Virus Disease (CEVD) / Koi Sleepy Disease (KSD)

Causative agent: Carp edema virus a large, double-stranded DNA virus belonging to the family *Poxviridae*.

Host: Common carp including Koi.

Clinical signs: Unresponsiveness and lethargy, with fish often lying motionless on their sides or bellies on the bottom of the pond is usually seen. If the "sleepy" carp or koi is disturbed, it may swim for a short period of time, but soon settle back into an inactive state on the bottom of the tank. Diseased juvenile koi may have extensive erosions or haemorrhages on the skin with edema of the underlying tissues. Other external signs of infection may include enophthalmos and pale swollen gills.

Transmission: Horizontal

1.5. Ranavirus disease

Causative agent: Ranaviruses, large-genome DNA viruses belonging to the family Iridoviridae.

Host: Koi, Seabass and marine ornamental fish

Clinical signs: Floating on water surface, sudden jerky movements, circling around the central axis, settling at the bottom of the tank, lethargy and anorexia.

Transmission: Horizontal

1.6. Tilapia Lake Virus (TiLV) disease

Causative agent: Tilapia Lake Virus (TiLV), single stranded positive sense RNA virus belonging to genus *Tilapinevirus* under family *Orthomyxoviridae.*

Host: Nile tilapia and Mossambique tilapia. All life stages are susceptible to TiLV.

Clinical signs: Lethargy, abdominal swelling, black discoloration, skin patches and ulceration, skin erosion, loss of scales, ocular alterations with opacity of lens/ exophthalmia.

Transmission: Horizontal and vertical.

1.7. Spring Viremia of Carp (SVC)

Causative agent: Spring Viraemia of Carp Virus (SVCV), RNA virus belonging to the genus *Vesiculovirus* under the family *Rhabdoviridae*.

Host: Carp (most susceptible), Goldfish, European catfish, Rainbow trout, Roach, Zebra fish

Clinical signs: Noticeable increase in mortality in the population, diseased fish usually appear darker in color. Exophthalmia, pale gills, haemorrhages on skin, abdominal distension, lethargy, loss of equilibrium, degeneration of gill lamellae, inflammation of intestine, oedema and hemorrhage of visceral organs are the other signs. Liver, kidney, spleen, gills and brain are the target organs

Transmission: Horizontal and vertical. Animate vectors and fomites may also be involved in transmission of SVCV. Temperature is the major predisposing factor.

1.8. Viral Encephalopathy and Retinopathy (VER)/ Viral Nervous Necrosis (VNN) disease

Causative agent: Betanodavirus, Negative sense single stranded RNA virus of the genus *Betanodavirus* under the family *Nodaviridae*.

Host: Mostly marine fish. Also reported in freshwater fish.

Clinical signs: VNN affects the nervous system. Brain, spinal cord and retina are the primary target organs. It also affects gonads. Abnormal swimming behaviour (cork-screwing, whirling, darting and belly-up motion) accompanied by variable swim bladder hyperinflation, cessation of feeding, changes in colouration, and mortality.

Transmission: Horizontal and Vertical

1.9. Viral Haemorrhagic Septicemia (VHS) disease

Causative agent: Viral haemorrhagic septicaemia virus (VHSV), negative stranded RNA virus belonging to genus Novirhabdovirus under the family Rhabdoviridae

Host: Marine and freshwater fish.

Clinical signs: Rapid onset of mortality (which can reach up to 100% in fry), lethargy, darkening of the skin, exophthalmia, anaemia (pale gills), haemorrhages at the base of the fins, gills, eyes and skin, distended abdomen and severe abnormal swimming behaviour, such as constant flashing and/or spiraling. The virus is abundant in all tissues including skin and muscles. Target organs are kidney, heart, spleen and brain.

Transmission: Horizontal.

1.10. Infectious Pancreatic Necrosis (IPN) disease

Causative agent: Infectious Pancreatic Necrosis Virus (IPNV) a double stranded RNA virus under the genus *Aquabirnavirus* of the family *Birnaviridae.*

Host: Freshwater and marine fish. IPN is an infection primarily of trout & salmon and the virus has also been isolated from a wide variety of other fish species.

Clinical signs: Mortalities can be as high as 90%. The virus is found to affect the pancreas and digestive system. Infected fry may show typical signs such as darkening of the skin colour, anaemia, anorexia, swimming high in the water column or lying on their side and hyperventilating and spiral swimming. Alimentary tract become filled with clear mucus, which contain sloughed epithelial cells and other cellular debris from intestinal wall. Ascites and exophthalmia may be seen in the affected fish. Liver and spleen are usually pale in colour.

Transmission: Horizontal and vertical

2. BACTERIAL DISEASES

2.1. Edwardsiellosis

Causative agent: *Edwardsiella tarda*

Host: Mostly cat fish, eels, mullets and tilapia

Clinical signs: Cutaneous lesions extending down to the musculature leading to necrosis of renal and hepatic tissue. Sometimes affected catfish hang in the water and show nervousness or symptoms of nervous breakdown. Exophthalmia and branchial anaemia may be the only external signs in the larger fish.

2.2. Bacterial gill disease

Causative agent: *Flavobacterium branchiophyllum*

Host: Mostly freshwater fish

Clinical signs: Attachment and proliferation of the filamentous bacteria on the gill mucoid surfaces, gasping, swelling of the opercula, and in severe cases, cotton wool like growth of bacteria.

2.3. Vibriosis

Causative agent: *Vibrio* spp.

Host: Brackishwater and marine fish

Clinical signs: Dark skin lesion which ulcerates to release the blood coloured exudate. The ulcers may be very deep and necrotic.

2.4. Bacterial haemorrhagic septicaemia

i) Causative agent: *Aeromona hydrophila*

Host: Freshwater and brackishwater fish. Most important cause of severe disease outbreak in cultured and wild freshwater fish.

Clinical signs: Affected fish show darkening in colour, with large red irregular haemorrhages on the body surface and base of fins and ascites. The haemorrhages on the skin surface may ulcerate to form shallow necrotic lesions. The skin lesions begin as severe oedema of the dermis and hyperaemia of the stratum reticulare, leading to spongiosis and ulceration of the epidermis followed by extensive haemorrhagic necrosis down the level of the muscle

ii) Causative agent: *Pseudomonas fluorescens*

Host: Freshwater and marine fish. Usually associated with concomitant environmental stress, especially high temperatures or overcrowding. Pond fish is most commonly affected but aquarium tropical fish, marine fish and salmonids may also succumb.

Clinical signs: Acute or chronic haemorrhagic septicaemia. Large haemorrhagic skin lesions are the most commonly observed signs and heavy mortalities may ensue very shortly after the advent of lesions. Cyprinid fish usually show ascites.

iii) Causative agent: *Photobacterium damsela ssp.* piscicida

Host: Marine fish

Clinical signs: Cause summer epizootics of 'pseudotuberculosis' with heavy mortality. Mass mortalities occur during the summer months. Affected fish are dark and anorexic, show generalised haemorrhagic septicaemia and reddening at the base of the fins

2.5. Streptococcosis

Causative agent: *Streptococcus iniae and Streptococcus agalatice*

Host: Marine and freshwater fish

Clinical signs: Generalized haemorrhages, corneal opacity, Exopthalmos and abscesses around the mouth. Internally enlarged spleen, abdominal distention and pale liver are seen. High stocking density, poor water quality conditions and high temperatures are the predisposing factors.

2.6. Mycobacteriosis

Causative agent: *Mycobacterium marinum and Mycobacterium fortuitum*

Host: Freshwater, brackish water and marine fish

Clinical signs: It is a chronic disease that may not produce clinical signs. External signs are scale loss and dermal ulcerations. Internally enlargement of spleen, kidney and liver with the presence of grey and white nodules.

3. FUNGAL DISEASES

3.1. Saprolegniasis

Causative agent: Water molds (oomycetes) mostly in the genus *Saprolegnia*.

Host: Mostly freshwater fish, incubating eggs

Clinical signs: White cotton like outgrowths of fungal hyphae are seen in the area of infection. Systemic infections produce mycelial masses in the gut and viscera causing peritonitis, extensive hemorrhage, necrosis and adhesions.

3.2. Branchiomycosis (Gill rot)

Causative agent: *Branchiomyces sanguinis* and *B. denigrans*

Host: Mostly freshwater fish

Clinical signs: Infected fish exhibit respiratory distress and a loss of equilibrium. The gill appears necrotic, eroded and pale, loss of gill lamellae

3.3. Epizootic Ulcerative Syndrome (EUS)

EUS is also known as red spot disease (RSD), Mycotic Granulomatosis (MG) and Ulcerative Mycosis (UM). The infection spreads rapidly and results in mass mortality.

Causative agent: *Aphanomyces invadans*is the necessary cause of the disease

Host: Murrels, Cat fish, Pearlspot and Mullets are more susceptible,

Clinical signs: In the early stage of the disease, red spots or small hemorrhagic lesions are generally found on the surface of fish. It progress to ulcers and eventually large necrotic erosions.

4. PROTOZOAN PARASITIC INFECTION

Ectoparasitic infestation may be suspected if fish shows irritability by flashing, rubbing against hard surfaces, excess mucus production, discolouration, gulping and sloughing of the epidermis.

4.1. White spot disease or freshwater Ich disease:

Causative agent: Protozoan ciliate ectoparasite, *Ichthyophthirius multifiliis.*

Host: It affects freshwater fish, mainly scaleless fish like catfish.

Clinical signs: The sites of infection are skin and gill. Parasites are visible as white grit like spots on skin, fins and gill. There will be excessive mucus secretion, erratic swimming patterns and rubbing the body against hard surface.

Transmission: Theront is the infective stage of the parasite that feeds on fish. High temperature, higher organic load and high stocking density are the predisposing factors for disease outbreak.

4.2. Trichodiniasis:

Causative agent: Protozoan ciliate ectoparasite, *Trichodina* spp.

Host: Freshwater, brackishwater and marine fish

Clinical signs: Main sites of infection are skin and gills. Infected fish may have darkened skin, greyish-blue coating over the body surface, progressive destruction of epidermis, irritability and excess mucus production.

Transmission: Higher organic load in the pond favours proliferation of the parasite leading to increased irritability and consequent behavioural change.

4.3. Ichthyobodiasis/ Costiasis/ Blue slime disease:

Causative agent: Protozoan flagellate ectoparasite, *Ichthyobodo necator*.

Host: Young salmonids and cyprinids especially in hatcheries.

Clinical signs: Main sites of infection are skin and gill. Infected fish secrete excess mucus that give dark grey or blue discolouration along the dorsal body wall. Raised nodules are seen on the body of fish.

Transmission: Horizontal

4.4. Hexamita infection or Hole in the head disease:

Causative agent: Endoparasitic flagellate protozoan, *Hexamita* spp*.*

Host: Commonly found in Oscar, Koi, Gold fish, Angel fish, Betta, Discus, Arowana, Clown fish, Surgeon fish and Parrot fish.

Clinical signs: Abdominal distension due to ascites, exophthalmia and whitish trailing faecal casts in the later stages. In some fishes like Oscar and Discus, formation of holes in the head is observed and it leads to secondary bacterial and fungal diseases. Faecal examination may reveal the presence of typical trophozoites.

Transmission: Trophozoite is the infective stage of the parasite that attacks fish. The primary site of infection is intestine and in advanced stages spreads to gall bladder and other internal organs. Transmission is horizontal.

5. HELMINTH PARASITIC INFECTION

5.1. Dactylogyrosis:

Causative agent: Monogenean parasite, *Dactylogyrus* spp. (Gill fluke)

Host: Mostly freshwater fish (Cichlids and Cyprinids)

Clinical signs: Inflamed and anaemic gill. Infected fish exhibit respiratory distress like gasping for air and often scratch against hard substratum. During microscopic examination of gill, small (<3 mm size) off-white flukes can be seen attached to gill filaments. It may also be seen on skin in some rare cases.

Transmission: Horizontal

5.2. Gyrodactylosis:

Causative agent: Monogenean parasite, *Gyrodactylus* spp. (Skin fluke)

Host: Freshwater and marine fish

Clinical signs: The main site of infection is skin but may also be seen on gills. Clinical signs are increased mucus production, frayed fins, skin ulcers, damaged gills and necrotic patches of skin epithelium.

Transmission: Horizontal

5.3. Black spot disease:

Causative agent: Metacercaria of digenean parasites, mainly *Diplostomum* spp.

Host: Freshwater and marine fish

Clinical signs: Metacercaria is the intermediate larval stage that infects the flesh of the fish resulting in black spots. Even though there is no mortality, the presence of black spots in the flesh reduces consumer acceptance.

Transmission: Fishes act as intermediate hosts in the life cycle of these trematodes. Management of this infection is through controlling molluscan primary host.

5.4. Cestode infection:

Causative agent: Cestodes (tape worms) are elongated and segmented endoparasites, visible to naked eye on necropsy. Some worms are big enough to fill the gut of the infected fish.

Host: Freshwater and marine fish

Clinical signs: Diseased fish will not take food and swims sluggishly at the surface. Mostly the fish are asymptomatic, however, infection leads to poor growth and chronic mortality.

Ligulosis, one of the cestode infections is caused by the intermediate plerocercoid stage of *Ligula intestinalis*. Migrating plerocercoids cause adhesions and severely damage viscera or muscles from pressure necrosis. Ichthyophagous birds are involved in outbreak of ligulosis.

Diphyllobothriasisis caused by fish tape worm, Diphyllobothrium latum, which is zoonotic. Main sites of infection are intestine, musculature and visceral organs. Clinical signs include floating on surface and head bent to one side due to muscular cramps and loosening of muscles due to encysted larvae. Some of the other important cestodes are Bothriocephalus sp. and Lytocestus sp.

Transmission: Horizontal.

5.5. Acanthocephalan infection:

Causative agent: Acanthocephalans (thorny headed worms). The parasite is characterised by the presence of an anterior spiny/ hooked proboscis for attachment. Numerous species of acanthocephalans infect fish. One of the major acanthocephalan infecting marine and brackishwater fish is *Tenuiproboscis keralensis*.

Host: Freshwater, brackishwater and marine fish

Clinical signs: Major clinical signs include reddening and inflammation of intestine and perforation of gut. The parasite does not cause severe mortalities, however, may lead to reduced growth.

Transmission: Horizontal. Generally amphipods act as intermediate host for acanthocephalans.

5.6. Nematode infection:

Causative agent: Nematodes are round worms. Adult parasite is mostly found in digestive tract, but may also inhabit peritoneal cavity, gonads and swim bladder. Penetration of parasite induces inflammation of affected internal organs.

Host: Freshwater, brackishwater and marine fish

Clinical signs: Reduced growth and weight loss are normally observed. In some cases, protrusion of the worms from the vent may also be seen.

Transmission: Horizontal. Avoid feeding organisms like live copepods and oligochete worms that harbour larvae.

6. LEECH INFESTATION

Causative agent: *Piscicola geometra.* It has anterior and posterior suckers. Leech attaches to fish with the help of suckers and feeds on the blood of fish. It secretes hirudin, which prevents blood clotting. After feeding it leaves the fish to digest its meal. Leeches act as vectors for blood parasites.

Host: Mostly freshwater fish

Clinical signs: The fish becomes anaemic and lethargic.

Transmission: Horizontal. Cocoons attach to leaves and other surfaces. (Highly resistant to chemical treatments)

7. CRUSTACEAN PARASITIC INFECTION

7.1. Argulosis:

Causative agent: *Argulus* spp. (fish lice)

Host: Freshwater and brackishwater fish

Clinical signs: Skin is the main site of infection. Fish skin is damaged by repeated piercing of stylet and the injection of toxic enzymes. This will cause irritation and leads to haemorrhages, hyper pigmentation, anaemia, secondary infection and ulcerative lesions.

Transmission: Horizontal. High temperature, higher organic load and high stocking density are the predisposing factors for disease outbreak.

7.2. Ergasilosis:

Causative agent: *Ergasilus* spp. (fish/ gill maggots)

Host: Freshwater and brackishwater fish

Clinical signs: Main site of infection is gill. Excessive mucus secretion, gasping for air, reduced feeding, erratic swimming.

Transmission: Horizontal. High temperature, higher organic load and high stocking density are the predisposing factors for disease outbreak.

7.3. Lernaeosis:

Causative agent: *Lernaea* spp. (anchor worm)

Host: Freshwater and brackishwater fish

Clinical signs: Main site of infection is skin, gills, fins and eyes. Haemorrhage, necrosis and inflammation at the site of attachment leading to secondary infection and emaciation.

Transmission: Horizontal. High temperature, higher organic load and high stocking density are the predisposing factors for disease outbreak.

8. ENVIRONMENTAL DISEASES

8.1. Gas bubble disease:

Cause: Super saturation of dissolved gases (nitrogen or oxygen)

Clinical signs: Bubbles in the abdominal cavity, eyes, skin, gills, fins, mouth, swimbladder and within the digestive tract and exophthalmia. Death due to embolism in blood and emphysema in tissues; edema and degeneration of the gill lamellae; bulging of the cornea; abrupt mass mortalities.

8.2. Nitrite poisoning or Brown blood disease:

Cause: Elevated levels of nitrite in the culture system water.

Clinical signs: The intensity of the condition can be evaluated by the color of the fish blood. Slightly affected fish have reddish-brown blood, whereas, more acutely affected fish have chocolate brown colored blood. Affected fish show signs of low oxygen stress even in the presence of saturated levels of dissolved oxygen.

8.3. Swim Bladder Stress Syndrome (SBSS):

Cause: Associated with malfunction of the swim bladder 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 super saturation during the day.

Clinical signs: Larvae show large bubble of gas in the region antero-dorsal to and outside the swim bladder. Hyperinflation of swimbladder; high positive buoyancy and mass mortalities.

8.4. Alkalosis:

Cause: Increase of pH to a level higher than the tolerance level.

Clinical signs: Corroded skin and gills and milky turbidity of the skin.

8.5. Acidosis:

Cause: Drop in the pH to a level lower than the tolerance level.

Clinical signs: Rapid swimming movements and gasping. Increased mucus secretion and death occur very quickly. The pond soil turns reddish in color. Normal metabolism is hindered resulting in retarded growth and eventual death.

8.6. Sunburn disease

Cause: Excessive levels of ultraviolet irradiation from sunlight when fish are stocked in shallow uncovered raceways under intense sunlight.

Clinical signs: Development of gray focal circular ulcerative lesions on top of the head, pectoral, dorsal and upper tail fins. Ulcerative lesions may serve as portals of entry for other pathogens and may result in secondary infection.

9. NUTRITIONAL DISEASES

9.1. Fish scoliosis/ Lordosis/ Spinal deformity/ Broken back syndrome

Cause: Deficiency of Vitamin C, Tryptophan, Magnesium, Phosphorus, essential fatty acids

Clinical signs: Lateral or dorso-ventral deformity

9.2. Lipidosis

Cause: Due to feeding with rancid feed or fatty/ poorly stored trash fish

Clinical signs: Poor growth, low mortality rate, lethargic movement, opaque eyes, slight distention of the abdomen and pale appearance of liver

9.3. EFA deficiency

Cause: Low levels of essential fatty acids in food

Clinical signs: Larval mortality known as "Shock Syndrome".

9.4. Nutritional myopathy

Cause: Due to rancid fat/ PUFA containing diets or deficiency of vitamin E.

Clinical signs: Body color darkening, emaciation, petechial at operculum and occasional spinal cord deformity.

9.5. Steatitis and white fat disease

Cause: Due to the deficiency of Vitamin E

Clinical signs: Fat deposition in the visceral cavity

9. 6. Clinical signs due to the deficiency of vitamin in fish:

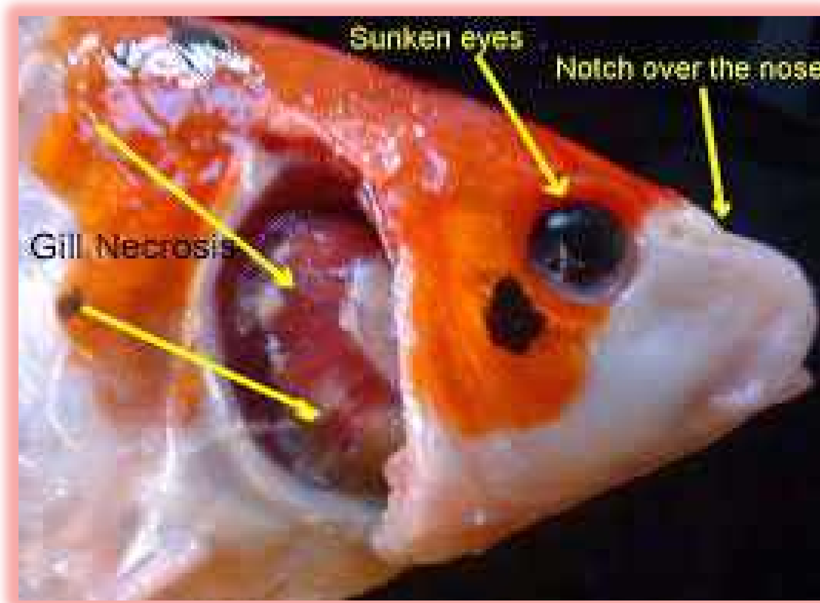
Vitamin	Deficiency Signs
Thiamine	Anorexia, poor appetite, muscle atrophy, loss of equilibrium, poor growth, congestion of fins and skin, fading of body color, lethargy.
Riboflavin	Eye and skin hemorrhage, photophobia, pigmentation of iris, striated constrictions of abdominal wall, dark coloration, anorexia, anemia, poor growth.
Pyridoxine	Nervous disorders, hyper-irritability, anemia, loss of appetite, rapid rigor mortis, peritoneal edema cavity, colorless serous fluid, rapid breathing, exophthalmia.

Pantothenic acid	Clubbed gills, necrosis, cellular atrophy of gills, gill exudate, loss of appetite, lethargy, poor growth, skin hemorrhage, skin lesions and deformities.
Niacin	Poor growth, anorexia, lethargy and mortality.
Inositol	Distended stomach, increased gastric emptying time, skin lesions, de-pigmentation and poor growth.
Biotin	Loss of appetite, lesions in colon, altered coloration, muscle atrophy, spastic convulsions and fragmentation of erythrocytes, skin lesions and poor growth.
Folic acid	Lethargy, fragility of caudal fin, dark coloration, macrocytic anemia, poor growth.
Choline	Poor food conversion, hemorrhagic kidney and intestine, poor growth, accumulation of neutral fat in hepatopancreas, enlarged liver.
Nicotinic acid	Loss of appetite, lesions in colon, jerky motion, weakness, edema, muscle spasms while resting, sensitivity to sunlight, poor growth, skin hemorrhage, lethargy and anemia.
Vitamin C	Impaired collagen formation, eye lesions, anorexia, hemorrhagic skin, liver, kidney and muscle; reduced growth, dark coloration, loss of balance, fin necrosis, high mortality
Vitamin A	Ascites, ceroid in liver, spleen and kidney; anemia, fragility of red blood cells, poor growth, exophthalmia, kidney hemorrhages, depigmentation and soft exoskeleton.
Vitamin D	Poor feed utilization, raised blood counts, slow growth rate, decreased ash levels, calcium and phosphorous, poor growth, soft exoskeleton, lethargy
Vitamin E	Muscular dystrophy, pathological condition in reproductive organs, increased permeability of capillaries, hemorrhages and edema in various parts of the body.
Vitamin K	Anemia, prolonged coagulation time.

9.7. Clinical signs due to the deficiency of major minerals:

Minerals	**Deficiency symptoms**
Calcium and Phosphorous	Slow growth rate and increased mortalities, scoliosis, lordosis and skull deformities.
Magnesium	Lordosis, poor growth and tetany.
Iron	Microcytic homochronic anemia
Manganese	Sluggish movement, loss of equilibrium, poor appetite, weight loss and mortality
Iodine	Thyroid, Hyperplasia (goiter)

1. VIRAL DISEASES OF FISH



1.1. Koi herpes virus disease (KHVD)

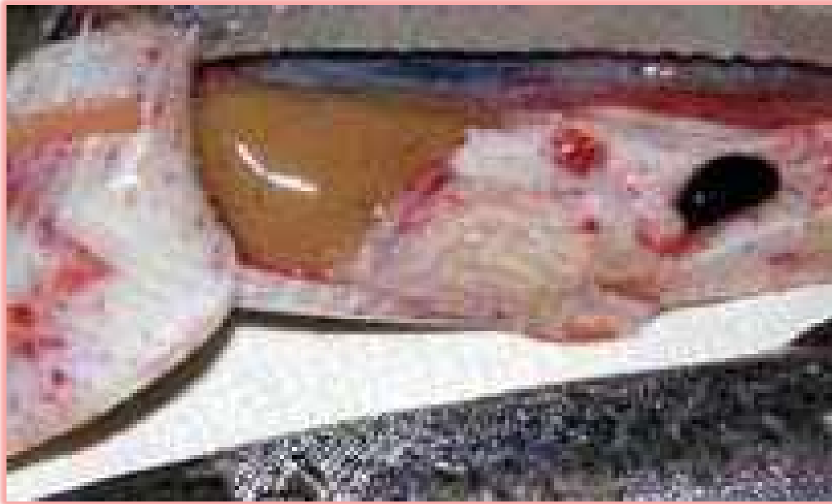
1.2. Congestion of brain in Sea bass with VNN

1.3.Carp edema virus-infected gills

1.4. SVC in common carp (haemorrhagic skin, swollen stomach and exophthalmos)

1.5. Noda virus in Lates calcarifer

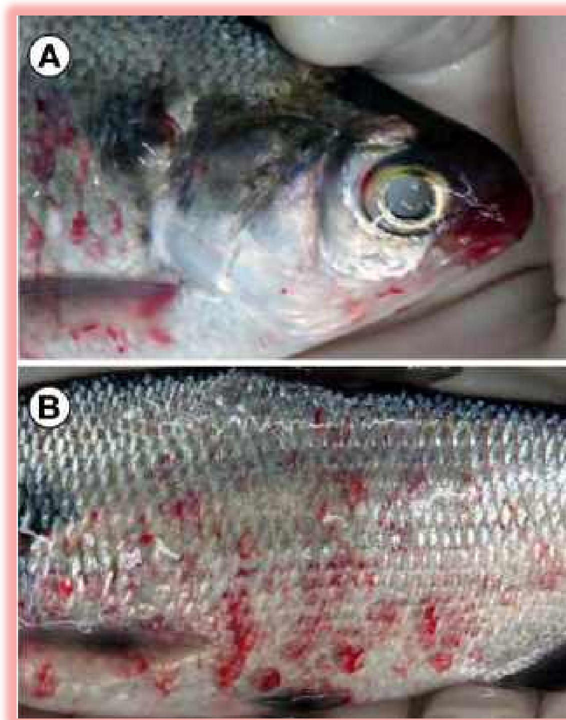
1.6. TiLV (Skin discoloration, erosion and loss of scales)



1.7. Viral haemorrhagic septicimia
(Hemorrhage of the muscle and internal organs)

1.8. Viral haemorrhagic septicimia- Exophthalmia

1.9.Bilateral exophthalmia and abdominal swelling



1.10. Petechial hemorrhages (A) around the mouth and (B) sides of the body

1.11. Hemorrhages in the pancreas in IPNV disease

2. BACTERIAL DISEASES OF FISH

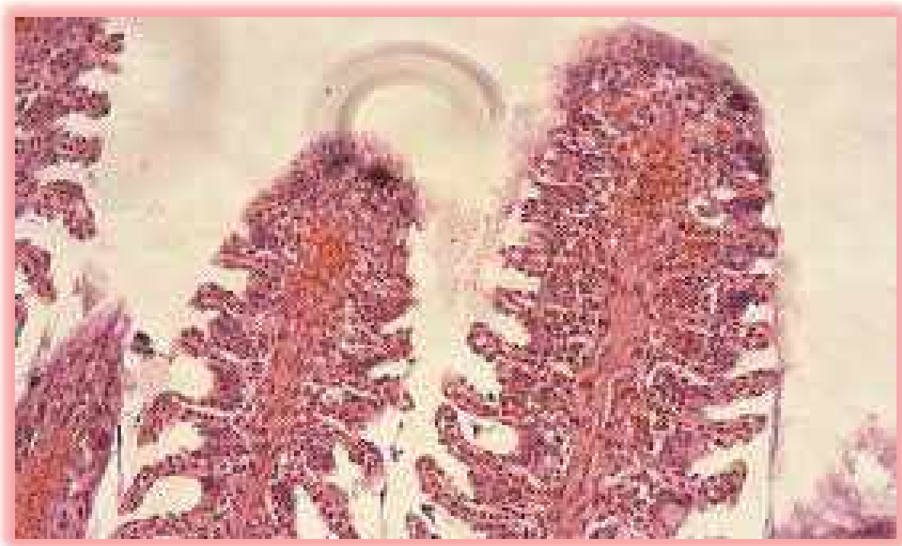
2.1. Fin rot and Tail rot

2.2. Dropsy

2.3. Eye infection

2.4. Vibriosis

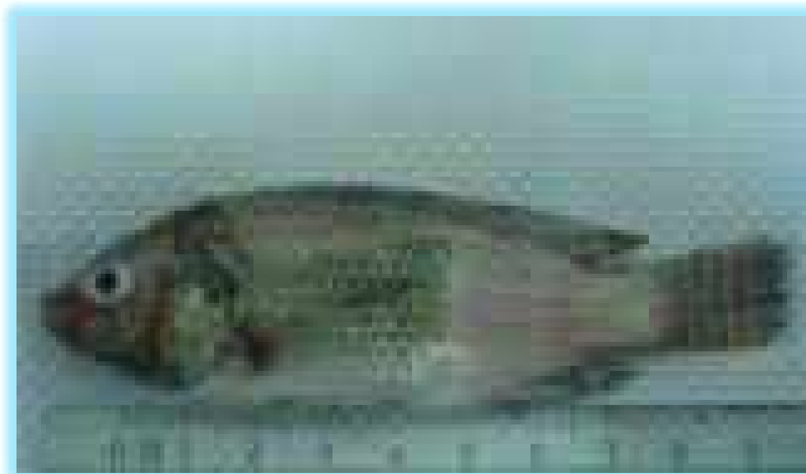
2.5. Gill infected with Flavobacterium



2.6. Gill hyperplasia

2.7. Ulcer

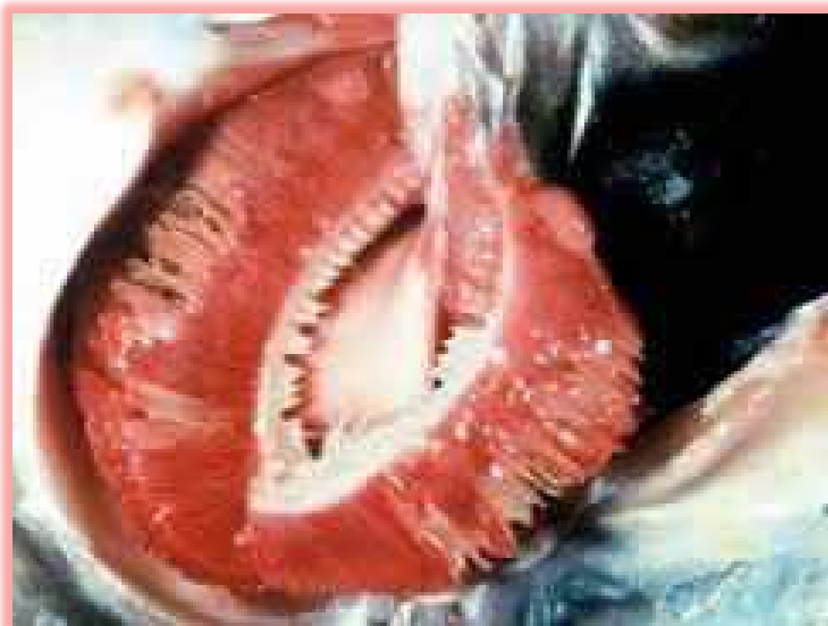
2.8. Bacterial gill disease



2.9. Columnaris disease

3. FUNGAL DISEASES OF FISH

3.1. Saprolegniasis

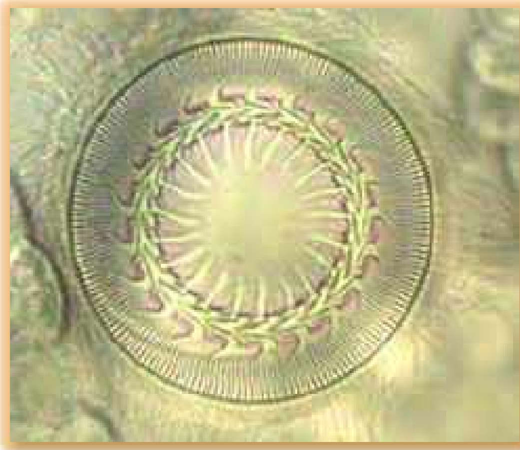


3.2. Branchiomycosis(Gill rot)

3.3. EUS affected fish

4. PARASITIC INFECTION OF FISH

4.1. Black moor infected with white spot



4.2. Trichodina under microscope

4.3. Koi infected with Gyrodactylus

4.4. Dactylogyrus

4.5. Fins with black spots

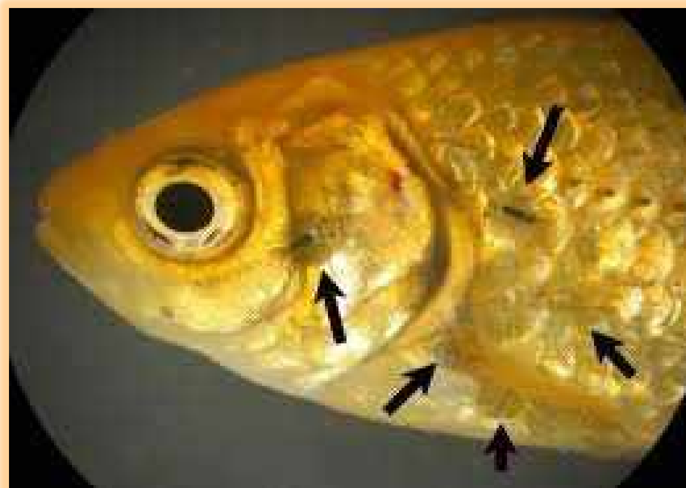
4.6. Ligula intestinalis

4.7. Fish gut infected with Acanthogyrus

4.8. Hole in the head disease

4.9. Koi showing Icthyobodiasis

5. CRUSTACEAN INFECTIONOF FISH



5.1 Argulus infection

5.2. Lernaea infection

5.3 Ergasilus on gills

6. NON INFECTIOUS DISEASES OF FISH

6.1. Gas bubble disease

6.2. Broken back syndrome

V. COMMONLY OCCURRING AND EMERGING SHRIMP DISEASES

1. VIRAL DISEASES

1.1. White-spot disease

Causative agent: White-spot syndrome virus (WSSV) belonging to the genus *Whispovirus* within the *Nimaviridae* family.

Host: Wide range of aquatic crustaceans (penaeids, crabs and crayfish).

Clinical signs: High mortality (up to 90–100% within 3–7 days post-infection), development of white spots on the exoskeleton, red discoloration of body and appendages, lethargy and sudden reduction in feeding. White spots may not occur in acute infection.

Transmission: Vertical and Horizontal

1.2. Monodon Baculo Virus (MBV) disease

Causative agent: Penaeus monodon nudivirus (PMNV)**/** Spherical baculovirus/ Monodon-type baculovirus (MBV) / Penaeus monodon Singly enveloped Nuclear Polyhedrosis Virus (PmSNPV) belonging to family *Nudiviridae*

Host: Penaeids.

Clinical signs: Pale-bluish-gray to dark blue-black colouration, sluggish and inactive swimming movements, loss of appetite and retarded growth. Presence of multiple spherical inclusion bodies in squash preparations of hepatopancreas and midgut epithelial cells.

Transmission: Horizontal

1.3. Infectious Hypodermal and Haematopoeitic Necrosis Virus (IHHNV) disease

Causative agent: Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV), Family *Parvoviridae*, Genus *Penstyldensovirus*. IHHNV is the smallest of the known penaeid shrimp viruses

Host: Penaeid shrimps

Clinical signs: Irregular, reduced growth and cuticular deformities, rather than mortalities, are found to be the principal effect of infection

Transmission: Horizontal and vertical

1.4. Baculovirus Midgut gland Necrosis Virus (BMNV) disease

Causative agent: Baculovirus midgut gland necrosis virus (BMNV) or Penaeus japonicus_non occluded baculovirus

Host: *Penaeus japonicus*

Clinical signs: The first gross sign of infection is the white, turbid appearance of the hepatopancreas. Severely affected postlarvae may float inactively on the surface of the water and display a white midgut line, mortalities in hatcheries occur in mysis to 20 day old postlarvae (PL) and may reach up to 98% in PL 9-10.

Transmission: BMNV is introduced to hatcheries with wild-caught brood stock and transmission to the larvae is thought to occur when the virus is shed with the faeces during spawning.

1.5. Penaeus monodon Hepandenso Virus / Hepatopancreatic Parvo Virus (HPV) disease.

Causative agent: Hepatopancreatic Parvovirus *(HPV)/* Penaeus monodon Hepandenso virus is a small parvo-like virus in family *Parvoviridae.*

Host: Penaeid shrimp

Clinical signs: Heavy infections cause poor growth without visible inflammatory response.

Transmission: Horizontal and vertical

1.6. Yellow Head Virus (YHV) disease

Causative Agent: Yellow Head virus (YHV)*,* a corona-like RNA virus in the genus *Okavirus*, family *Roniviridae*.

Host: Primarily *Penaeus monodon*

Clinical signs: The cephalothorax of infected shrimp turn yellow after a period of unusually high feeding activity ending abruptly, and the moribund shrimps congregate near the surface of the pond before dying. YHD leads to death of animals within 2 -4 days

Transmission: Horizontal

1.7. Taura Syndrome Virus (TSV) disease

Causative agent: Taura syndrome virus (TSV), a small picorna-like RNA virus that has been classified in the new family *Dicistroviridae*.

Host: Primarily *Litopenaeus vannamei.*

Clinical signs: Pale red body surface and appendages, tail fan and pleopods particularly red, shell soft and gut empty, death usually at moulting, multiple irregularly shaped and randomly distributed melanised cuticular lesions

Transmission: The most likely route for transmission of TSV is cannibalism of dead infected shrimp. The virus can be spread from one farm to another by seagulls and aquatic insects, shrimp surviving a TSV infection are lifelong carriers of the virus and are a significant source of virus for susceptible animals.

1.8. Infectious Myonecrosis Virus (IMNT) disease

Causative Agent: Infectious Myonecrosis Virus (IMNV) is an emerging shrimp RNA virus

Host: Primarily *Penaeus vannamei*

Clinical signs: Affected shrimp present extensive white necrotic areas in the striated muscle, especially of the distal abdominal segments and tail fan. These may become necrotic and reddened in some individual shrimp.

Transmission: Horizontal via water and by cannibalism

1.9. Macrobrachium rosenbergii Noda Virus (MrNV)/ White tail disease

Causative Agent: Macrobrachium rosenbergii nodavirus (MrNV) and Extra small virus (XSV)

Host: *Macrobrachium rosenbergii* (larvae, PL and early juveniles are susceptible, whereas adults are resistant)

Clinical signs: Lethargy and opaqueness of the abdominal muscle. Opaqueness appeared at the centre of the abdominal muscle and gradually extended anteriorly and posteriorly with 100% mortality within 2–3 days after appearance of the whitish muscle.

Transmission: Vertical and horizontal

2. BACTERIAL DISEASES

2.1. Acute Hepatopancreatic Necrosis Disease

Disease usually characterized by mass mortality during first 35 days of culture, thus named as Early mortality syndrome (EMS)

Causative agent: *Vibrio parahaemolyticus* having Pir toxin gene in its plasmid

Host: *Penaeus monodon, L. vannamei, and P. chinensis*

Clinical signs: Affected shrimp have slow growth rate, spiral swimming, empty gut, hepatopancreatic discolouration due to loss of connective tissue, and black discolouration due to melanization

2.2. Vibriosis

Primary and secondary infection by *Vibrio* spp. leads to vibriosis. Disease is also called Systemic or enteric vibriosis, Sea gull syndrome, Splinters

Causative agents: *Vibrio parahaemolyticus, V. harveyi*

Host: Larvae, post larvae, juveniles and adults of penaeids

Clinical signs in hatchery: High to severe mortalities, reduced or absence of faecal strands, delayed moulting, haemocytic inflammation and melanization

Clinical signs in grow-out farm: Reduced feeding, high to severe mortalities, increase in clotting time, hepatopancreas with white/ black streaks, cloudiness in hepatopancreas in PL, cloudiness of muscles in sixth abdominal segment, and progressive spots on gills and lymphoid organ

2.3. Bacterial Shell disease

This disease is also called as brown spot shell disease/ burned spot disease/ rust disease/ shell disease or black spot disease.

Causative agent: *Vibrio, Pseudomonas*

Clinical Signs: Lesions in brownish to black colour, single or multiple eroded areas on the general body cuticle, appendages, or gills, lesions begin as small local lesions (due to abrasions, puncture wounds, chemical trauma or other causes) that rapidly enlarge.

2.4. Brown or Black Spot disease (Rust disease, Burned Spot disease)

Causative agents: *Vibrio, Aeromonas* and *Pseudomonas.*

Host: Larvae, post larvae, juvenile and adult of penaeids

Clinical signs: Appearance of brownish to black erosion of the carapace, abdominal segments, rostrum, tail, gills and appendages. Affected part shows a cigarette butt like appearance; Infection starts at sites of injuries or from other damage caused by cannibalism, lesions progress upon entry and multiplication of bacterial pathogens.

2.5. Mycobacterial infections

Causative agent: *Mycobacterium marinum*

Clinical signs: Abnormally dark pigmentation in areas of the body that hard multicolour melanised haemocytic nodules or larger prominent melanized granulomatous lesions composed of multiple nodules occur. Lesions observed in the lymphoid organ, heart, cuticle and systemically in the loose connective tissue of muscle, hepatopancreas, antennal gland, ovary and gills.

3. FUNGAL DISEASES

3.1. Hepatopancreatic microsporidiasis

Causative agent: *Enterocytozoon hepato Penaei*(EHP), a microsporidian parasite. It is an obligate spore forming intracellular pathogen within the fungal kingdom.

Host: Mostly *L.vannamei* and *P. monodon*

Clinical signs: The pathogen infects the hepatopancreatic tissue of shrimp leading to unusual growth reduction and differential growth. The disease can be diagnosed by the presence of spores in the hepatopancreas and faecal matter by wet smears and histology of hepatopancreas.

Transmission: Horizontal, mainly by oral route. EHP can be prevented only by good management practices and adopting bio-security measures. Once the spores are in the pond, it is very difficult to eradicate. Proper pond preparation including drying and disinfection between each culture operation must be practiced to remove the spores. Application of quick lime (CaO) @ 6 tons/ha to a pH of 12 during pond preparation and removal of bottom debris to kill the spores and control infestation is recommended. During culture, organic load of the pond needs to be controlled, which in turn minimizes the spore density preventing infestation.

3.2. Larval mycosis

Causative agent: *Lagenidium* spp., *Sirolpidium* spp., *Haliphthoros* spp

Host: Eggs, larvae and early postlarvae of all Penaeids and crabs.

Clinical signs: Sudden onset of mortalities in larval stages of shrimps and crabs. Characteristic fungal hyphae give whitish appearance on the body.

3.3. Black gill disease (Fusarium disease)

Causative agent: *Fusarium solani*

Host: Penaeids. Largely associated with broodstock. *Penaeus japonicus* and *P. stylirostris* are particularly susceptible while *L.vannamei* appears fairly resistant

Clinical signs: Brownish or blackish gills. Appearance of "black spots" that preceded mortalities in juvenile shrimps

4. ENVIRONMENTAL, NUTRITIONAL AND IDIOPATHIC DISEASES

4.1. Muscle necrosis

Cause: Temperature and salinity shock, low oxygen levels, overcrowding, rough handling and severe gill fouling.

Clinical signs: Opaque white areas on the abdomen; 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. 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.

4.2. Bent/ cramped tails or body cramp in shrimps

Cause: Handling of shrimp in warm, humid air much warmer than culture water, and mineral imbalance.

Clinical signs: Partial or complete rigid flexure of the tail. 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.

4.3. Incomplete moulting

Cause: Due to low temperature of culture water

Clinical signs: Presence of old exoskeleton attached to newly moulted larvae, especially in appendages. Abnormal swimming movement which could lead to easy predation and mortality.

4.4. Black gill disease

Cause: 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).

Clinical signs: The gills of affected shrimps show reddish, brownish to black discoloration and, in advanced cases, gill filaments become totally black. Histological observations show that blackening of the gills may be due to the deposition of melanin at sites of tissue necrosis and heavy haemocyte activity.

4.5. Red discolouration of shrimp

Cause: Associated with higher application of lime in the pond that gives it a high initial pH or prolonged exposure to low salinity.

Clinical signs: Red short streaks on gills or abdominal segments, yellowish to reddish discoloration of the body and increased fluid in the cephalothorax, emitting foul odour. Yellow to red discoloration in affected shrimps; histopathology of the hepatopancreas shows haemocyte 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.

4.6. Chronic soft-shell syndrome

Cause: Associated with exposure of normal hard-shelled shrimps to pesticide or piscicide.

Clinical 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. Affected shrimps are soft-shelled, grow slowly, and eventually die

4.7. Acidosis:

Cause: Drop in the pH to a level lower than the tolerance level.

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

4.8. Aflatoxicosis (Red disease)

Causative agent: Aflatoxin B1 produced by *Aspergillus flavus* and other *Aspergillus* spp. which are common contaminants of improperly stored or expired feeds

Host: All shrimps.

Clinical signs in fish: Pale gills, anaemia, impaired blood clotting and mortality in heavy doses. Chronic exposure to lower dose cause tumours in liver and kidney.

Clinical signs in shrimp: Yellowish, and eventually reddish discoloration of the shrimp body and appendages can be observed among pond-cultured shrimp juveniles. Other signs include lethargy, weak swimming activity near pond dikes, soft shelling, retarded growth and lesions in hepatopancreas and mandibular organ.

1. VIRAL DISEASES OF SHRIMP

1.1. White spot virus infected shrimp

1.2. Taura syndrome

1.3. Infectious myonecrosis

1.4. White tail disease

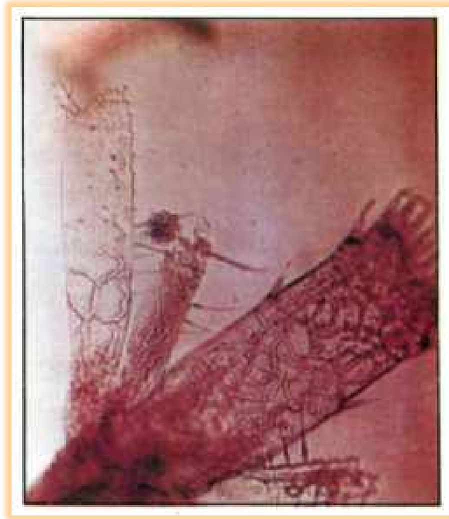
1.5. Yellow head virus in shrimp

2. BACTERIAL DISEASES OF SHRIMP

2.1. Vibriosis in shrimp

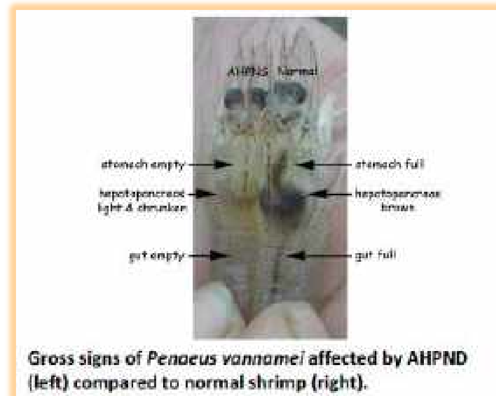
2.2. Mycobacterial infection

2.3. Black gill disease (Fusarium disease)



2.4. Larval mycosis

2.5. Black spot disease



Gross signs of *Penaeus vannamei* affected by AHPND (left) compared to normal shrimp (right).

2.6. Acute hepatopancreatic necrosis

3. PROTOZOAN DISEASES OF SHRIMP

3.1. Hepatopancreatic microsporidiasis

4. NON INFECTIOUS DISEASES OF FISH

4.1. Bent/cramped tails or body cramp

VI. AQUATIC ANIMAL HEALTH LABORATORY PROTOCOLS

1. ANALYSIS OF WATER QUALITY PARAMETERS

1.1. Total Alkalinity

Reagents

- Na_2CO_3 solution (0.02N): Dissolve 0.108 g of sodium carbonate (Na_2CO_3) in distilled water and make up to 100ml.
- H_2SO_4 (0.02N): Dilute 6 ml of conc. H_2SO_4 (35N) with DW under cooling and make up to 1L with in a volumetric flask. This stock gives 0.2N H_2SO_4. Dilute 100ml of this 0.2N H_2SO_4 with 1000ml DW to get a 0.02N H_2SO_4 in a volumetric flask. Standardise this acid against a standard solution of sodium carbonate.

  To find out exact normality of H_2SO_4, following equation can be used :

  $$\text{Normality of } H_2SO_4 = \frac{\text{Normality of } Na_2CO_3 \times \text{Vol. Of } Na_2CO_3}{\text{Vol. of } H_2SO_4 \text{ consumed}}$$
- Phenolphthalein indicator: Dissolve 0.25g of phenolphthalein powder in 50 ml of 50% alcohol.
- Methyl orange indicator (aqueous): Dissolve 0.01g methyl orange and 0.05 g of bromocresol green in 100 ml of water. Colour change at end point (pH 4.3) is sharper than that shown by methyl orange alone.

Procedure

- ✓ Take 100 ml of filtered sample in a conical flask and add few drops of alcoholic phenolphthalein indicator;
- ✓ Titrate the solution with 0.02 N H_2SO_4 if a pink colour develops, until it becomes colourless and note the titre value. This is the phenolphthalein alkalinity (PA);
- ✓ Add a few drops of aqueous methyl orange indicator and titrate against the acid till the end point (blue green to orange) and note the titre value. It is total alkalinity (TA);
- ✓ The nature of alkalinity can then be predicted from the titration as follows:-

Titration end point	Nature of alkalinity
Phenolphthalein end point is zero	Bicarbonate alone
No further titration to the methyl orange end point after the phenolphthalein end point	Hydroxides alone

Phenolphthalein end point titration is half the total titration	Carbonate alone
Phenolphthalein end point titration is greater than half the total titration	Both carbonate and hydroxides
Phenolphthalein end point is less than half the total titration	Both carbonate and bicarbonates

Calculation:

$$CO_3^{2-} \text{ (mg L}^{-1}) = \frac{(2X) \times \text{Normality of } H_2SO_4 \times 30 \times 1000}{100}$$

$$HCO_3^{-} \text{ (mg L}^{-1}) = \frac{(Y - 2X) \times \text{Normality of } H_2SO_4 \times 61 \times 1000}{100}$$

Volume of water sample taken = 100 ml

Volume of 0.02 N H_2SO_4 used for phenolphthalein end point = X ml

Volume of 0.02 N H_2SO_4 used for methyl orange end point = Yml

Volume of 0.02 N H_2SO_4 required for neutralize the carbonate alone = 2X ml

Volume of 0.02 N H_2SO_4 required for neutralize the bicarbonate alone = (Y – 2X)

Normality of H_2SO_4 = 0.02 N

1.2.Hardness

Reagents: Ammonia buffer solution, Eriochrome black T (EBT) indicator and Standard EDTA titrant (0.01 M)

Procedure:

- ✓ Preparation of 0.01 M EDTA: Weigh 3.723 g of dipotassium EDTA and transfer it to a 1000 ml standard flask. Make the solution up to the mark using distilled water. Shake the solution well for uniform concentration.

Titration of water sample

- ✓ 50 ml of dilute water sample is pipetted out into a clean dry conical flask;
- ✓ Add 2 ml of ammonia buffer solution to the sample;
- ✓ Add 1-2 drops of Eriochrome black T indicator to the above solution;
- ✓ Titrate the contents against standard EDTA taken in the burette until the last reddish tinge disappears;
- ✓ Add drops of EDTA carefully till the solution turns blue (indicates the end point);
- ✓ Note down the volume of EDTA consumed.

Calculations

$$\text{Hardness (mg/L of } CaCO_3) = \frac{V1\ X M X equivalent weight of CaCO3\ x\ 1000}{V}$$

V1= Volume of EDTA consumed (ml)

V = Volume of sample (ml)

M= Molarity of EDTA

Equivalent weight of $CaCO_3$ = 50

1.3.Dissolved Oxygen (DO)

Reagents

- Winkler- A (WA): Dissolve 400 g $MnCl_2$ in 1000 ml DW and store in a polyethylene bottle.
- Winkler- B (WB): Dissolve separately and mix together, 360 g KI and 100 g NaOH in 1000 ml DW and store in a polyethylene bottle.
- HCl (50%): Carefully add 50 ml conc. HCl to 50ml DW and store in a ground stoppered glass bottle.
- Sodium thiosulphate solution (0.02 N): Dissolve 5 g sodium thiosulphate in DW and make up to 1000 ml in a volumetric flask. Store in a ground stoppered glassbottle.
- Starch solution (indicator): Disperse 1 g starch in 100 ml hot DW, quickly heat the suspension to boiling (complete dissolution of starch) and cool and store in a ground stopper glass bottle. This solution should not be kept for more than a week.
- Standard iodate solution (0.02 N): Dissolve 0.3567 g potassium iodate (AR) in 500 ml DW in a volumetric flask.

Procedure

(a) Reagent blank:

- ✓ Pipette out 50 ml DW in 100 ml conical flask and add 3 ml of 50% HCl and mix well;
- ✓ Add 1 ml of WB and mix;
- ✓ Add 1 ml of WA and again mix well;
- ✓ Add 1 ml starch indicator to get blue colour and titrate against thiosulphate from the burette and note down the burette reading when blue colour disappears;
- ✓ Repeat the experiment three times and find out the mean burette reading [BR (b)].

(b) Standardization of thiosulphate solution (follow the steps in same order of (1)):

- ✓ Add 10 ml of 0.02 N KI solution, mix well and keep in dark for 5 minutes to allow iodine to liberate; Titrate liberated iodine with sodium thiosulphate solution from the burette till the solution turns pale yellow;
- ✓ Add 1ml of starch indicator and continue titration drop by drop till the colour changes from blue to colourless and remains colourless atleast for 30 seconds;
- ✓ Repeat the experiment three times and find out the mean burette reading [BR (st)].

$$\text{Normality of sodium thiosulphate N} = \frac{10 \times 0.02}{\text{BR (st)} - \text{BR (b)}}$$

(c) Sample Analysis:

- ✓ Add 3 ml of 50% HCl, by inserting the pipette tip close to the settled precipitate in DO bottle. Stopper the bottle immediately and shake vigorously till all precipitate dissolves;
- ✓ Pipette out 50ml of the clear solution in the conical flask and titrate against thiosulphate solution from the burette using starch as given above and note down the burette reading [BR (s)].

(d) Calculation

$$DO\ (ml/L) = 5.6 \times N \times [BR_{(s)} - BR_{(b)}] \times \frac{V}{[V-1]} \times \frac{1000}{a}$$

DO (mg/l) = DO (ml/L)/0.7

N = Normality of thiosulphate

BR(s) = Titre value of the sample (mean)

BR (b) = Titre value of the blank (mean)

V = Volume of the sample bottle (125 ml)

a = Volume of the sample titrate (50 ml)

1.4. Biological Oxygen Demand (BOD)

Reagents

- ➢ Phosphate buffer solution: Dissolve 0.85 g KH_2PO4, 2.175 g K_2HPO_4, 3.34 g $Na_2HPO_4.7H_2O$ and 0.17 g NH_4Cl in about 50 ml DW and dilute to 100 ml. [pH should be 7.2].
- ➢ $MgSO_4$Solution: Dissolve 0.225 g $MgSO_4.7H_2O$ in DW and dilute to 100 ml.
- ➢ $CaCl_2$ Solution: Dissolve 2.75 g $CaCl_2$ in DW and diluted to 100 ml.
- ➢ $FeCl_3$ Solution: Dissolve 0.025 g $FeCl_3.6\ H_2O$ in DW and dilute to 100 ml.
- ➢ Winkler A: Dissolve $MnSO_4.4$ H2O in filtered DW and diluted to 100 ml.
- ➢ Winkler B: Dissolve 70 g KOH and 15g KI in DW and diluted to 100 ml. Dissolve 1 g of NaN_3 (Sodium Azide) in 4 ml DW and added to the former.
- ➢ Concentrated Sulphuric acid.
- ➢ Starch indicator: Dissolve 2 g laboratory grade soluble starch in DW and slightly boil to dissolve. [Prepare freshly].
- ➢ Sodium thiosulphate solution: Dissolve 25 g $Na_2S_2O_3$ in DW and add 0.4 g NaOH (to arrest bacterial decomposition) and dilute to 1000 ml.

Procedure

- ✓ Prepare dilution water by adding 1 ml of phosphate buffer, $MgSO_4$, $CaCl_2$ and $FeCl_3$ each to 1 L DW and saturate with air;
- ✓ Dilute the contaminated water sample with dilution water and note the dilution factor;
- ✓ Fill the diluted water sample in BOD bottles carefully and stopper tightly;
- ✓ Keep one bottle in BOD incubator for 20^0 C for 5 days or 27^0 C for 3 days (final);
- ✓ Determine the DO of one bottle (Initial) immediately: Add 1 ml winkler A and 1 ml winkler B solutions into water sample taken in BOD bottle and stopper the bottle carefully;
- ✓ Invert the bottle several times carefully to ensure thorough mixing of reagents and a brown precipitate indicate the presence of DO;
- ✓ Keep the sample bottle for several minutes and allow the precipitate to settle;
- ✓ After reaching a clear zone on the top, re-stopper the bottle and add 1 ml conc.H_2SO_4;
- ✓ Stopper the bottle carefully and mix the contents well by inverting several times to get clear yellow solution;
- ✓ Pipette out 20 ml of the above solution in to a clean dry conical flask and titrate against 0.01 N Sodium thiosulphate taken in the burette;
- ✓ When solution turns pale yellow, add few drops of starch indicator (solution turns to blue) and continue titration carefully until to a colourless end point;
- ✓ Repeat the titrations to get concordant values.

Calculations

Correction factor (*f*) for sample volume = $\frac{(V-X)X20}{V}$

V= Volume of BOD bottle

X = Volume of reagent consumed

Dissolved oxygen content of the water sample= $\frac{8XVol\ of\ thiosulphateX1000}{f}$

BOD of the water sample = DO initial – DO final

1. 5. Ammonia – Nitrogen

Reagents

- ➢ Phenol alcohol: Dissolve 10 g of phenol in a mixture (100 ml) consisting of ethyl alcohol (95 ml) and n- propanol (5 ml).
- ➢ Sodium nitroprusside: Dissolve 1 g of sodium nitroprusside in 200 ml of MQ water and store it in an amber glass bottle.

- Sodium citrate: Dissolve 20 g of sodium citrate and 1 g of NaOH in 100 ml of MQ water.
- Sodium hypochlorite: Commercially available Sodium hypochlorite ("Chlorox") of 1.5 N.
- Oxidizing reagent: Mix 100 ml of sodium citrate solution and 25 ml of sodium hypochlorite solution just before use.

Preparation of standard solution:

- ✓ Dissolve 0.5349 g of Ammonium chloride (AR) in 100 ml of MQ water. This solution contains 10,000 µmol/L.(Stock solution);
- ✓ Transfer 1 ml of the above 10,000 µmol solution into another 100 ml volumetric flask and dilute to the mark with ammonia free MQ water. It contains 100 µmol/L;
- ✓ Transfer 1ml of the above 100 µmol solution into another 100 ml volumetric flask and dilute to the mark with ammonia free MQ water. This solution contains 1 µmol/L.

0.5349 g in 100ml = 10,000 µmol/L
1 ml from 10,000 µmol solution in 100 ml = 100 µmol NH_4 -N /L.
1 ml from 100 µmol solution in 100 ml = 1 µmol/L.
2 ml from 100 µmol solution in 100 ml = 2 µmol/L.
3 ml from 100 µmol solution in 100 ml = 3 µmol/L.

Calibration of standard and blanks:

- ✓ Measure out 50 ml of working standard solutions in clean stopper glass tubes in triplicate and 50 ml of MQ water in clean stopper glass tubes as blank;
- ✓ Add 2 ml of phenol alcohol solution and mix well;
- ✓ Add 2 ml of sodium nitroprusside solution followed by 5 ml of oxidizing solution and mix well once again and cover the flasks with polythene sheet and wait for 1 hour;
- ✓ Measure the absorbance of std. solution A(st) and blank A(b) in a spectrophotometer using 1 cm cell at 640 nm against ammonia free MQ water as reference.

Sample analysis:

- ✓ Measure out 50 ml of the sample (triplicate) in a clean stopper glass tube and add 2 ml of phenol alcohol solution and mix well;
- ✓ Add 2 ml of sodium nitroprusside solution followed by 5 ml of oxidizing solution and mix well and cover the flasks with polythene sheet and wait for 1hour;
- ✓ Measure the absorbance of sample in a spectrophotometer using 1 cm cell at 640 nm against ammonia free MQ water as reference.

Calculation:

- ✓ Factor value (F) = Conc. of standard solution / A(st) - A(b)

  A (st) = Mean absorbance of standards.

  A (b) = Mean absorbance of blanks.

- ✓ Ammonia µmol/L = F x A(s) – A (b)

  Where A(s) = Mean absorbance of samples.

  A(b) = Mean absorbance of blanks.

1.6. Nitrite – Nitrogen (NO2-N)

Reagents

- Sulphanilamide: Dissolve 1 g of sulphanilamide in 10 ml conc. HCl and make up to 100 ml with MQ water and store in an amber glass bottle.
- N – (1- naphthyl) – Ethylene diamine dihydrochloride (NEDA): Dissolve 0.1 g of NEDA in 100 ml of MQ water and store it in an amber glass bottle.
- Nitrite stock solution**:** Anhydrous sodium nitrite ($NaNO_2$) is dried at 110°C for some hours. Weigh accurately 0.069 g of $NaNO_2$ and dissolve in 100 ml of MQ water up to the mark in 100 ml standard flask. This stock solution contains 10 m mol/L NO_2 - N (or) 10,000 µmol/L NO_2 -N.

Procedure

Working standard solution:

- ✓ Transfer 1 ml of nitrite stock solution into a 100 ml volumetric flask and dilute to the mark with MQ water. This solution contains 100 µmol NO_2 –N/L.
- ✓ Again transfer 1 ml of the above 100 µmol solution into another 100 ml volumetric flask and dilute to the mark with MQ water. This solution contains 1 µmol NO_2 -N/L. Prepare similarly for following standards as 2, 3, 4 and 5 µmol NO2-N/L concentrations from above said stock solution.

  0.069 g in 100 ml = 10,000 µmol NO_2 -N/L

  1 ml from 10,000 µmol solution in 100 ml = 100 µmolNO_2 -N /L

  1 ml from 100 µmol solution in 100 ml = 1 µmolNO_2 -N/L

  2 ml from 100 µmol solution in 100 ml = 2 µmolNO_2 -N/L

  3 ml from 100 µmol solution in 100 ml = 3 µmolNO_2 -N/L

Calibration of standards and blank:

- ✓ Measure 25 ml of MQ water for blank in triplicate;
- ✓ Measure 25 ml of working std. solution in clean stopper glass tube in triplicate;
- ✓ Add 0.5 ml of sulphanilamide to each tube and mix well;

- ✓ Add 0.5ml of NEDA solution after 1 min reaction time, mix well once again and allow to proceed for 15 min;
- ✓ Measure the absorbance of blank A(b) and standard solutions A(st) in a spectrophotometer using 1 cm cell at 540 nm against MQ water as reference.

Sample analysis:

- ✓ Measure out 25ml of the sample (triplicate) in a clean stopper glass tube and add 0.5 ml of sulphanilamide to each tube and mix well;
- ✓ Add 0.5 ml of NEDA, mix well and wait for 15 min;
- ✓ Measure the absorbance A (s) of the sample in 1 cm cell at 540 nm.

Calculation

$$\textbf{Factor value (F)} = \frac{\text{Conc. of standard solution}}{A(st) - A(b)}$$

A (st) = Mean absorbance of standards

A (b) = Mean absorbance of blanks.

NO2 –N μmol/L = F x A(s) – A (b)

A(s) = Mean absorbance of samples.

A(b) = Mean absorbance of blanks.

1.7. Iron content

Reagents

- ➢ Conc. HCl
- ➢ Hydroxylamine solution- Dissolve 10 g NH_2OH. HCl in 100 ml DW
- ➢ Ammonium acetate buffer solution- Dissolve 250 g $NH_4C_2H_3O_2$ in 150 ml water, add 700 ml concentrated (glacial) acetic acid and dilute to 1L
- ➢ Phenanthroline solution – Dissolve 100 mg 1,10-phenanthroline monohydrate $C_{12}H_8N_2.H_2O$ in 100 ml water by stirring and heating to 80^oC. Do not boil. Discard the solution if it darkens. (*Note: heating is unnecessary if 2 drops conc. HCl is added to wastewater, 1 ml of this reagent is sufficient for no more than 100 mg Fe)*
- ➢ Stock iron solution (prepare freshly): Slowly add 20 ml conc. $H_2SO_4$to 50 ml water and dissolve 1.404 g $Fe(NH_4)_2(SO_4)_2$. $6H_2O$, add 0.1 M Potassium Permanganate drop wise until a faint pink colour persists and dilute to 1000 ml with distilled water (1 ml = 200 mg Fe).
- ➢ Standard iron solutions (prepare daily for use):

  10 mg Fe – 100 mg Fe

  Pipette 50 ml stock solution into 1000 ml volumetric flask and dilute to mark with distilled water

1 ml = 10.0 mg Fe

(1 ml, 2 ml, ..., 10 ml)

1 mg Fe- 10 mg Fe

Pipette 5 ml stock solution into a 1000 ml volumetric flask and dilute to mark with distilled water

1 ml = 1.00 mg Fe (1 ml, 2 ml, ..., 10 ml)

Procedure

- ✓ Take 50 ml water sample in a clean, dry 20 ml conical flask (dilution required for concentrated sample);
- ✓ Add 2 ml conc. HCl and 1 ml NH_2OH. HCl solution;
- ✓ Add a few glass beads and heat to boiling;
- ✓ Continue boiling till volume is reduced to 15 to 20 ml to ensure dissolution of iron;
- ✓ Cool to room temperature and transfer to a 50 ml or 100 ml volumetric flask;
- ✓ Add 10 ml $NH_4C_2H_3O_2$ buffer solution;
- ✓ Add 4 ml phenanthroline solution and dilute to the mark with distilled water;
- ✓ Mix thoroughly and allow a minimum of 10 min for maximum colour development;
- ✓ Set the spectrophotometer at 510 nm and measure blank and set zero absorbance;
- ✓ Measure standards with increasing concentrations;
- ✓ Plot a calibration curve including blank.

Calculation

Amount of iron in water sample can be calculated from the standard graph using the absorbance value of water sample in mg/L.

2. ANALYSIS OF SOIL PARAMETERS

2.1. Organic matter

Reagents

- ➢ 1 N $K_2Cr_2O_7$ solution : Weigh 24.5 g of oven dried (130° C for 2 hrs) $K_2Cr_2O_7$ and dissolve in DW and dilute to 500 ml in a standard flask
- ➢ Ferrous Ammonium Sulphate (FAS) – 0.5 N: Dissolve 98 g of FAS in 300 ml DW containing 20 ml conc.H_2SO_4 and dilute to 500 ml standard flask
- ➢ Ferroin indicator: Dissolve 1.485 g 1,10 phenanthroline monohydrate and 695 mg of $FeSO_4.7H_2O$ in DW and dilute to 100 ml.

Procedure

- ✓ Weigh 0.2 g of sieved soil and transfer to a dry 500 ml conical flask;
- ✓ Pipette out 10 ml of 1 N $K_2Cr_2O_7$ in to the same flask;
- ✓ Add 20 ml conc. H_2SO_4 slowly with mixing and swirl the flask gently and leave on a dry surface for 30 min;
- ✓ Add 200 ml DW followed by 3 drops of ferroin indicator;
- ✓ Titrate against 0.5 N FAS taken in the burette;
- ✓ Note the colour change from bluish green to red;
- ✓ Repeat titration to get concordant values.

Calculation

% of Organic Carbon = $\frac{(A-B)*N*100}{w*100}$

(A- Titre value of blank, B- Titre value of soil sample, N- Normality of FAS, W- weight of soil)

% of Organic matter content in soil = % C X 1.724

2.2. pH

Reagents: Buffer solutions of pH 4, 7 and 9

Procedure

- ✓ Weigh 10 g soil, dried and sieved;
- ✓ Add 100 ml DW and stir with glass rod;
- ✓ Switch on the pH meter and allow to warm up;
- ✓ Calibrate the meter with buffer solutions;
- ✓ Collect the supernatant from soil solution and dip the clean dry electrodes to it;
- ✓ Note down the readings from pH meter.

3. MICROBIOLOGICAL TECHNIQUES FOR ISOLATION OF BACTERIA

3.1. Preparation of Media

Liquid broth media

- ✓ Weigh the prescribed quantity of powder (as mentioned on the label of the pack) and dissolved in the required amount of water in a conical flask;
- ✓ Dissolve the ingredients by warming, poured into test tubes and sterilized in autoclave.

Solid plate/ slant/ deep tube media

- ✓ Weigh the prescribed quantity of powder and dissolved in required amount of water in a conical flask;
- ✓ Sterilize the prepared medium in autoclave and then allowed to cool for some time;
- ✓ Poured in to sterilized petridishes or test tubes while still warm and before solidifies;
- ✓ Allow to solidify upon cooling to room temperature.

  (Note: Media in very hot condition should never be poured into containers, as it leads to condensation of water on the wall of the containers, which drop on the surface of the media and may lead to its contamination).

3.2. Sterilisation

- ✓ Glasswares are sterilized in hot air oven at 160^0C for 1 hour;
- ✓ Media in autoclave at 121^0C (15 psi pressure) for 15 minutes.

  (Note: Glass wares can also be sterilized in autoclave, but media should never be sterilized in the oven, as water escapes from the media and they dehydrate).

3.3. Inoculation

- ✓ A definite volume of the homogenous liquid sample is pipetted into the broth/ solid agar plate aseptically. (In the case of solid agar plate, spread the sample aseptically on the surface of the medium);
- ✓ In case of solid samples, a sample of definite weight is mixed aseptically with normal physiological saline (0.85% NaCl) in the ratio of 1:9 (1g+9ml) using sterilized pestle and mortar or a blender and the homogenized liquid suspension is used for inoculation;
- ✓ In case of surface samples (body surfaces or wounds), it is rubbed with a sterilized swab and the swab is touched to the surface of a sterilized nutrient agar/ TSA plate and from the point of touch, streaks are made by sterilized loop aseptically.

3.4. Incubation

- ✓ The inoculated broth tubes and plates are incubated at 37^0C room temperature for 24 to 48 hrs.

3.5. Observation

- ✓ Turbidity in liquid broth and colony growth on agar plates indicate growth of bacteria.
- ✓ On observation, if no colony growth has taken place on the agar plate, the sample used is bacteria free while growth is found, there is presence of bacteria.

3.6. Isolation

- ✓ Select representative colonies (colonies having dissimilar characteristics) of bacteria from the mixed population

- ✓ Isolate the individual species of bacteria aseptically by inoculating onto separate agar plates to get the pure cultures as per the standard protocol.
- ✓ Observe and analyse the inoculated plates for colony characteristics:
  - ➢ Size : Pinpoint, small, moderate or large
  - ➢ Pigmentation : Colour of colony
  - ➢ Form : The shape of the colony is described as follows
    - (a) Circular : Unbroken peripheral edge
    - (b) Irregular : Indented peripheral edge
    - (c) Rhizoid : Root like spreading growth
  - ➢ Margin : The appearance of the outer edge of the colony is described as follows
    - (a) Entire : Sharply defined, even
    - (b) Lobate : Marked identifications
    - (c) Undulate : Wavy indentations
    - (d) Serrate : Tooth like appearance
    - (e) Filamentous : Thread like spreading edge
  - ➢ Elevation : The degree, to which colony is raised as described as follows
    - (a) Flat : Elevation not discernible
    - (b) Raised : Slightly elevated
    - (c) Convex: Dome shaped elevation
    - (d) Umbonate: Raised with elevated convex central region

4. EXAMINATION OF AQUATIC ANIMAL FOR FUNGUS

4.1. Wet mount staining

Lactophenol Cotton Blue (LCB) wet mount staining method is widely used for fungi. Ingredients required per litre for LCB preparation is given below:

Cotton blue	:	0.5g
Phenol crystals	:	200g
Glycerol	:	400ml
Lactic acid	:	200ml
Distilled water	:	200ml

Procedure

- ✓ Place a drop of lactophenol cotton blue reagent on a clean and dry slide;
- ✓ Tease carefully the fungal culture/ squash preparation of lesions/ scales/ gills by using a sterilised nichrome inoculating wire or tooth pick and keep it in the dye solution;
- ✓ Place a cover slip on the preparation and wait for about 5 min;
- ✓ Observe under microscope.

5. MOLECULAR DIAGNOSIS OF AQUATIC ANIMAL

5.1. Extraction of genomic DNA

Reagents:

- 10% Sodium lauryl/ dodecyl sulphate (SDS): Add 10 g Electrophoresis grade SDS in 100 ml distilled water by heating the solution to 68°C. Adjust pH to 7.2. Sterilize by sterile filter, if needed to store at RT.
- Proteinase K: Add 20 mg Proteinase K in 0.5 ml sterile DW and 0.5 ml molecular biology grade Glycerol and store at -20°C.
- NaCl (5M): Add 29.22 g NaCl in 100 ml DW
- Chloroform & Isoamyl alcohol (24:1): Prepare the solution containing 24 ml chloroform and 1 ml Isoamyl alcohol and store at 4° C.
- Phenol, Chloroform & Isoamyl alcohol (25:24:1): Prepare the solution containing 25 ml Phenol (Tris-Cl saturated, pH 8.0), 24 ml Chloroform and 1 ml Isoamyl alcohol and store in a dark bottle at 4° C.
- 70% ethanol: Add 100 ml absolute alcohol in 42 ml DW
- Lysis Buffer: Add 250 µl 2M Tris, 2000 µl 0.5 M EDTA and 1000 µl 10% SDS into 10 ml DW

Procedure:

- ✓ Dissect the gills and/ or pleopod and chop them into fine pieces using sterile scissors;
- ✓ Transfer 100 mg of finely minced pieces of samples into a micro-centrifuge tube;
- ✓ Add lysis buffer containing Tris-EDTA and SDS (1 ml for 100 mg shrimp tissue) and macerate the tissue finely;
- ✓ Add proteinase K @ 10 µg/ml and incubate at 65°C for 2 hours;
- ✓ Add equal volume of phenol, chloroform and isoamyl alcohol and centrifuge at 9000 g for 5min;
- ✓ Recover the supernatant and add an equal volume of chloroform and isoamyl alcohol;
- ✓ Mix and centrifuge at 9000 g for 5 min;
- ✓ Recover the supernatant and add 0.1 vol of 5M NaCl and 2 vol of absolute ethanol;
- ✓ Centrifuge at 12,000 g for at 10 min and discard the supernatant;
- ✓ Wash the pellet with 70% ethanol and centrifuge at 12,000g for 10 min;
- ✓ Remove the supernatant and dry the pellet by placing the microfuge tubes inverted over a tissue paper in laminar airflow chamber;
- ✓ Dissolve dried DNA pellet in 50 µl 1XTE buffer or sterile deionized DW;
- ✓ Use the extracted DNA as template for PCR (If required, storeat 4°C for short term storage or at -20°C for long term storage).

5.2. Polymerase Chain Reaction (PCR)

It is a technique that employs *in-vitro* enzymatic amplification of a particular or desired DNA fragment up to several kilobases (kb) in size from a complex genome. A thermo stable DNA polymerase (Taq polymerase) isolated from a thermophilic bacterium,*Thermus aquaticus*, is most often used and since it is heat stable, it does not have to be replaced after each cycle. PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. The region to be amplified is defined by hybridization of two specific oligonucleotides to the DNA molecule, one to each strand of the double helix. The oligonucleotides are known as the forward and the reverse primers. In PCR, the double stranded DNA is denatured by heat and then the temperature is lowered to allow annealing of two specific primers by complementary base pairing on the opposite strands of the DNA. Taq polymerase directs the synthesis of the new strand from the primed sites in both directions that results in double stranded DNA and the procedure is repeated 30 times. In each cycle, the target DNA is replicated by a factor of 2 so that, after 30 cycles millions of copies of DNA are available.

Reaction mixture:

- The amount of template DNA for a total reaction mixture of 50 µl should be in the range of 0.01-1 ng for plasmid/ phage and 0.1-1 µg for genomic DNA.
  (Higher amount of template DNA increase the yield of nonspecific PCR products).
- Primer should have 15-30 nucleotides in length and the GC content should be 40-60%.
  (More than three G or C nucleotides at the 3' end of the primer should be avoided, as nonspecific priming may occur. The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation).
- The concentration of $MgCl_2$ should be 1-4 mM.
  (Too few Mg_2+ ions result in a low yield of PCR product, and too many increase the yield of non-specific products and promote misincorporation, as Mg_2+ ions form complexes with dNTPs, primers and DNA templates.)
- The concentration of each dNTP in the reaction mixture should be 200 µM.
  (Inaccuracy in the concentration of even a single dNTP (dATP, dCTP, dGTP, dTTP) dramatically increases the mis-incorporation level).
- The range of Taq DNA polymerase should be 1-1.5 units in 50 µl of reaction mix.
  (Higher Taq DNA polymerase concentration leads to synthesis of non specific products).

Procedure for reaction mixture preparation:

- ✓ Prepare a master mix containing water, buffer, dNTPs, primers, Taq DNA polymerase and $MgCl_2$ in a single tube to perform several parallel reactions, which can then be aliquoted into individual tubes (This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers);
- ✓ Template DNA solution is added after aliquoting into tubes;
- ✓ Gently vortex and briefly centrifuge all solutions after thawing;
- ✓ Add the following in a thin-walled PCR tube placed on ice;

Reagent	Final concentration	Quantity, for 50µl of reaction mixture
Sterile deionized water	-	Variable
10X Taq buffer	1X	5µl
10mM dNTP mix	0.2mM of each	1µl
Primer I	0.1-1µM	Variable
Primer II	0.1-1µM	Variable
Taq DNA Polymerase	1.5 u	Variable
25mM $MgCl_2$	1-4mM	Variable*
Template DNA	10pg-1µg	Variable

* Table for selection of 25 mM $MgCl_2$ solution volume

Final concentration of $MgCl_2$ in 50µl reaction mix, in mM	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, in µl	2	2.5	3	3.5	4	5	6	8

- ✓ Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube;
- ✓ Place samples in a thermocycler and start PCR.

Procedure for Thermocycling

- ✓ Perform the initial denaturation at 95°C over an interval of 1-3 min, if the GC content is 50% or less (This interval should be extended up to 10 min for GC-rich templates).
- ✓ Perform denaturation at 94-95°C for 0.5-2 min (PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and is completely denatured under this condition).

- ✓ Calculate annealing temperature of the primers using the formula,

  Tm = 4 (G + C) + 2 (A + T)

  Annealing temperature (^{0}C) = Tm – 5^{0}C

  Tm = Melting temperature; G, C, A, T = number of respective nucleotides in the primer
- ✓ Perform initial extension at 72°C for 1 min to synthesis PCR fragments up to 2 kb (The rate of DNA synthesis by Taq DNA Polymerase is highest at this temperature).
- ✓ The number of PCR cycles would be 25-35 depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product.
- ✓ Perform final extension at 72°C for 5-15 min after the last cycle to fill in the protruding ends of newly synthesized PCR products.

5.3. Visualization of PCR products by Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate DNA strands by size and to estimate the size of the separated strands by comparison to fragments of known size (DNA ladder). Agarose gel matrix is acting as a sieve, through which DNA fragments moves when electric currentis applied. The dye used for the visualization of DNA bands in agarose gel electrophoresis is ethidium bromide (EtBr) which has the unique property of fluorescing under UV light when intercalated with DNA. By running DNA through an EtBr-incorporated gel and exposing it to UV light, distinct bands of DNA become visible. Loading buffers are added with the DNA in order to keep track of the position of DNA during electrophoresis and to sediment it in the gel well. Xylene cyanol and Bromophenol blue are generally used. They run along with 4-5 kb and 400-500 bp DNA fragments respectively in 0.5-1.5% agarose gel. There are a number of buffers used for agarose electrophoresis. The most common are Tris acetate EDTA (TAE), Tris Borate EDTA (TBE) and Sodium borate (SB) buffer.

Reagents required

- ➢ TAE buffer (50X): Add 242 g Tris base, 57.1 ml Glacial acetic acid and 100 ml EDTA (0.5 M, pH, 8.0) and make up the solution to 1 litre using distilled water and sterilize by autoclaving at 121^{0}C for 15 min. A 1X TAE buffer for electrophoresis is prepared by diluting 20 ml of stock to 1L using DW.
- ➢ Gel loading buffer (6X): Add 0.25 g Bromophenol blue, 0.25g Xylene cyanol FF and 40g Sucrose in 100 ml distilled water and stored at 4°C.
- ➢ Ethidium bromide (EtBr**)**: Dissolve 5 mg of EtBr in 1 ml of DW. The solution may either be added to agarose during preparation or to the staining medium (1X TAE buffer) after the run, to yield a final concentration of 0.5 µg/ml. In the latter case, allow the

gel to stain in the staining solution for 20 min and destain in DW for 10 min to remove excess stain. Observe the gel on UV transilluminator.

Procedure

- ✓ Weigh required amount of agarose and add required volume of 1XTAE. (Usually the percentage of agarose ranges between 0.7-2.0% depending on the size of DNA to be electrophoresed)
- ✓ Boil the solution in a microwave oven/hot plate till the solution completely clears.
- ✓ Allow the solution to cool down to about 60-65 °C at room temperature.
- ✓ Meanwhile, set up the gel rack and insert the comb at one side of the rack.
- ✓ Wear gloves and add EtBr at a concentration of 0.5 µg/ml. (Caution:ethidium bromide is a potent mutagen).
- ✓ Mix the solution gently, avoiding air bubbles, to disperse the EtBr, then fill it into the gel rack, keeping the thickness of the gel at around 0.5-0.9 cm.
- ✓ When the gel has cooled down and become solid, remove the comb. The holes that remain in the gel are the slots/ wells.
- ✓ Put the gel, together with the rack, into a chamber with 1XTAE. Make sure the gel is completely covered with TAE buffer.
- ✓ Load the wells with 10 µl of DNA mixed with 2 µl of loading buffer. Load one well with a DNA ladder. Close the lid of the electrophoresis chamber and apply voltage/ current (typically, 100 V for 30 min with 15 ml of gel). When the "dye front" approaches the end of the gel, the current is stopped.
- ✓ Visualize the DNA (stained with ethidium bromide) under a transilluminator.
- ✓ (Caution: Wear goggles/protective gear to avoid exposure to UV light)

6. AQUATIC ANIMAL HEALTH LABORATORY

Aquatic Animal Health Laboratories (AAHL) would be in a three tier system at State, Regional and Sub-district levels. The State laboratory would function as the referral laboratory with capabilities to analyse all disease related parameters of aquatic animal and would include components such as microbiology, molecular diagnosis, biochemistry, histopathology units and analytical facilities including facility for detection of heavy metals, antibiotic residues, pesticides, poly aromatic hydrocarbons (PAH) and a wet laboratory. The Regional laboratory would have soil and water analytical units, microbiology and molecular diagnostic units.

At sub-district level, there would be mobile Laboratory with sufficient equipments and consumables for analysis of soil and water quality, sample/ smear preparation, examination of parasites and necropsy and would also carry portable generator set, oxygen cylinder, microscope, balances, small refrigerator, etc.

7. COMPONENTS OF AAHL

7.1. Water and soil analysis unit: - It shall have facilities for performing the basic water quality testing along with the facility for storage of samples and reagents, the equipment such as refrigerators, spectrophotometer, pH meter, redox meter, electronic weighing balance, DO meter, oven, water bath, salinometer, multiparameter water analysis kit/ probe, BOD Incubator, glasswares/ plasticwares, and other consumables required for soil and water analysis.

7.2. Microbiology unit: - It shall be available in state and regional level laboratories. Microbiology laboratory should have physically separated working area for media preparation, sample preparation, necropsy, air conditioned inoculation and incubation rooms, sterilization and decontamination rooms in an unidirectional work flow. Each type of the analysis should have dedicated equipments to avoid cross contamination within and between working areas. It should have all the adequate equipment such as Bio Safety Cabinet Class II Type B2 (total exhaust), BOD incubator, carbon dioxide incubator, circulating water bath, digital colony counter, pH meter, frost free double door refrigerator, fumigator, hot air oven, incubators of variable ranges viz., ambient to 70°C & 5°C to 50°C, lab blender, laminar air flow, filtration assembly, micropipette sets for variable volumes, orbital shaker/water bath, refrigerated centrifuge, trinocular microscope with attached digital camera and documentation unit, upright laboratory (manual defrost), vertical deep freezer (-25°C) & -80°C, UV viewing chamber, UV-vis spectrophotometer, water bath – serological along with minor equipments such as vortexer, manual colony counter, bell jars, anaerobic jar, analytical balance, autoclave, automated culture media preparator with pourer stacker, automated glassware washer, cleaning, furnitures etc and other consumables including protective clothing.

7.3. Histopathology unit: - It shall be available in state level laboratory. Histopathology unit would have instruments viz., automatic tissue processor, tissue embedder, microtome, water bath, hot plate, hot air oven, staining setup, refrigerator, microscope with imaging and recording and all other minor consumables.

7.4. Molecular diagnostic unit: - It shall be available in state and regional level laboratories. It would have physically separated working area for pre-PCR, PCR and post-PCR work to avoid contamination and cross-contamination between the samples in each step. It would have adequate dedicated equipments and protective clothing and consumables. It would have all the equipments to be fully functional

viz., thermal cycler, real time PCR (for state laboratory), PCR hood, laminar air flow, deep freezer (-25°C) and ultra low freezer (-80°C), water bath and dry bath, refrigerated micro centrifuge, ice flaking machine, water purification system, high precision electronic weighing balance, pH meter, vortexer, temperature controlled magnetic stirrer, microwave oven, UPS, horizontal electrophoresis system with power pack, gel documentation system with computer and dedicated micropipettes sets for each working area.

7.5. Biochemistry unit: - It shall be available in state level laboratory capable of detecting pesticide, antibiotic residue, mycotoxins, heavy metal and poly aromatic hydrocarbons-PAH. It would have the additional facilities such as sample extraction room, ELISA readers, ICP-MS, LC-MS/MS, GC-MS/MS, HPLC, FTNIR, microwave digestion units, spectrophotometer, flash evaporator with chiller, hot air oven, analytical balance, automatic serum analyser, muffle furnace, crucibles, kjeldahl and soxhlet apparatus, other minor equipments and consumables.

7.6. Cell culture unit:- This facility available at state level shall have an air conditioned room with equipments such as biosafety cabinet class-II, inverted trinocular fluorescent microscope with digital imaging, ultracentrifuge, refrigerated centrifuge, cooled incubator, deep freezer, refrigerators, liquid nitrogen containers, micropippetes, automatic pippeter and necessary consumables including disposable plasticware. In addition, the cell culture unit would have sterilization equipment such as autoclave, hot air oven etc.

7.7. Wet Laboratory: - It shall be available in state level lab and certain selected regional labs. There shall be provision for fibre tanks with aeration/ oxygenation, isolation containment with decontamination facility, fresh /sea water storage tanks, water treatment and filtration units.

7.8. Others: - There shall be waste disposal unit, drainage with effluent treatment plant, incinerator, UPS, generator, furniture and fixtures, solar water heater, rain water harvesting system, fire extinguishers and solar power unit. The working process in the entire laboratory shall be unidirectional starting with sample receiving cell, samples coding, sample storage, handing over to the analyst, conducting the test requested, result despatch after decoding the sample details.

8. HUMAN RESOURCE REQUIREMENT FOR AQUATIC ANIMAL HEALTH LAB

a) The State referral laboratory would have Director, Quality Manager, Technical Manager (Biological), Technical Manager (Chemical), Technical Officer (Microbiology), Technical Officer (Biochemistry), Technical Officer (Biotechnology), Technical Officer (Fisheries), Technical Officer (Aquatic Animal Health Management) and Lab Assistants for each unit.

b) Each regional laboratory would have a Quality manager, Technical Manager, Technical Officer (Microbiology), Technical Officer (Aquatic Animal Health Management) and Lab Assistants for each component.

c) There would be one clinical practitioner (Fisheries) at sub district level who shall look after the mobile laboratory.

d) The qualifications for the HR shall be as prescribed below:

No	Category	Essential qualifications
1	Director	• Post graduate degree in Aquatic Animal Health Management/ Fish Pathology/ Fish Microbiology/ Aquatic Environment Management/ Fish Biotechnology/ Fish Biochemistry; • PhD in any one of the above field; • Must have minimum 5 year experience in any of the above field.
2	Quality Manager	• Post graduate degree in Aquatic Animal Health Management/ Fish Pathology/ Fish Microbiology/ Aquatic Environment Management/ Fish Biotechnology/ Fish Biochemistry; • Must have minimum 5 year experience in any of the above field.
3	Technical Manager (Biological)	• Post graduate degree in Aquatic Animal Health Management/ Fish Pathology/ Fish Microbiology/ Fish Biotechnology; • Must have minimum 3 year experience in any of the above field.
4	Technical Manager (Chemical)	• Post graduate degree in Aquatic Animal Health Management/ Aquatic Environment Management/ Fish Biochemistry; • Must have minimum 3 year experience in any of the above field.
5	Technical Officer	• Post graduate degree in Aquatic Animal Health Management/ Fish Pathology/ Fish Microbiology/ Aquatic Environment Management/ Fish Biotechnology; • Must have minimum 3 year experience in any of the above field.
6	Clinical practitioner	Bachelor of Fisheries Science (4 years duration) from a Agricultural university or Fisheries university.
7	Lab Assistant	VHSE in any Fisheries subjects.

VII. THERAPEUTIC APPLICATION

Treatment or therapy is intended to restore the health of the diseased or infected animal. Based on the nature of the etiological agent, suitable chemicals/ drugs can be administered in the required doses for their control. The dose and method of administration should be selected based on the environmental condition, species, size, condition of the animal and the drug delivery. The treatment should be done under constant observation and should be discontinued in case of any sign of distress. The efficiency of the medicine is influenced by water hardness, pH and temperature.

Treatment of diseased fish in an extensive pond is practically difficult and is also expensive, risky and labour intensive; however it can be recommended for intensive culture practices. Therapeutics are generally used to control diseases. However, antibiotics are not usually recommended in aquaculture practices because of the formation of harmful residues and development of drug resistant bacteria or pathogens.

1. USE OF THERAPEUTICS OTHER THAN ANTIBIOTICS

These chemicals are used to control infections due to microbial and parasitic agents as and when disease is suspected. Personnel who are involved in the application of medicine should wear gloves.

1.1. Bacterial infection of fish

Disease/ Pathogen	Level	Drug/Chemical	Method of administration	Dosage
Flexibacter, Flavobacteria, Aeromonas, Pseudomonas, etc (for external infection)	1	NaCl	LTB	5-10 ppt for 5-7 days
			STB	10-20 ppt for 30 minutes
	1	Chloramine T	STB	18-20 ppm
	1	Benzalkonium chloride (BKC)-50%	STB	0.5-1 ppm for several hours 10 ppm for 5 -10 minutes
	1	$CuSO_4$	Dip	0.5 ppm for 1-2 minutes (toxic under low alkalinity)
	1	$KMnO_4$+Lime	LTB	250 g +10 kg per acre mixed with 10 kg sand(should apply during dusk only)
	2	Formalin	STB	0.05 - 0.35 ml per litre for 10 minutes with an interval of once in two hours
Bacteria (Surface disinfection of eggs)	1	Iodophores (10% iodine) Effectiveness reduce in alkaline conditions	STB	0.5- 1 ppm free 10 minutes
			Dip	25 ppm for 5 minutes. In poorly buffered water (< 50 ppm total alkalinity), add 1 g of sodium bicarbonate/ liter to unbuffered povidone iodine solutions; otherwise, they will lower the pH and kill eggs.

1.2. Fungal infection of fish

Disease/ Pathogen	Level	Drug/ Chemical	Method of administration	Dosage
Aphanomyces invadans (EUS)	1	NaCl	LTB	More than 3 ppt
	2	Lime + Turmeric (9:1)	LTB	10kg+1kg per acre
	2	CIFAX	LTB	0.01 ppm for 7-14 days
	2	Formalin	STB	0.25 ml/ litre for 30 minutes
	2	Formalin	LTB	0.025 ml/ litre
Saprolegniasis	1	NaCl	LTB	5-10 ppt
	2	H_2O_2	STB	500 ppm for 15 minutes (for eggs) once per day

Branchiomycosis and others	1	Benzalkonium chloride (BKC)-50%	STB	0.5-1 ppm for several hours 10 ppm for 5 -10 minutes
	2	$CuSO_4$ (toxic under low alkalinity)	STB	0.25-1 ppm
			Dip	2-5 ppm
	2	Formalin	STB	10 ppm for 30 minutes for 7 days
	2	Malachite green	Immersion	50-60 ppm for 10 - 30 seconds
			STB	1 ppm for 1 - 1.5 hours
			LTB	0.2- 0.5 ppm for 6 days
			Combined LTB	0.1 ppm malachite green and 25 ppm formalin for every other day for 3 days with aeration

1.3. Protozoan parasites of fish

Disease/ Pathogen	Level	Drug/Chemical	Method of administration	Dosage
Cryptobia, Ichthyobodo, Chilodonella, Trichodina and *Trichodinella*	1	NaCl	STB	5-10 ppt for 30 minutes OR 10-20 ppt for 15 minutes
		Ivermectin	Oral	50ug/ kg body weight, two times at an interval of one week
		$KMnO_4$	STB	50 ppm for 15 minutes
		Chloramine-T	STB	18-20 ppm for 1 hour
	2	Formalin	STB	250 ppm (0.25 ml/ litre) for 30 minutes
			LTB	25 ppm (0.025ml/ litre)
Ichthyophthirius multifiliis	1	Formalin	LTB	25 ppm (0.025ml/ litre)
	2	Malachite green oxalate	STB	50-60 ppm for 10 to 30 seconds
			STB	1 ppm for 1 - 1.5 hours
			LTB	0.2 - 0.5 ppm for 6 days
			LTB	0.1 ppm malachite green and 25 ppm formalin for every other day for 3 days with aeration

1.4. Helminth parasites of fish

Disease/ Pathogen	Level	Drug/Chemical	Method of administration	Dosage
Monogeneans (*Dactylogyrus, Gyrodactylus) The occurrence should be informed to State surveillance team immediately*	1	Ivermectin	Oral	Feed 50 g/kg body weight once. Repeat if necessary after 14 days.
		H_2O_2	STB	300 ppm for 10-15 minutes
	2	Formalin	STB	250 ppm for 30 minutes
			LTB	25 ppm (0.025 ml/ litre)
	2	Praziquantel	LTB	2 ppm for 24 hours
	2	Mebendazole	LTB	1 ppm for 24 hours
Digeneans/ Trematodes	1	Praziquantel	STB	10 ppm for 1 hour
			Oral	330 mg/ kg body weight once
Cestodes	1	Praziquantel	LTB	2 ppm for 1-3 hours
			Oral	50 mg/ kg body weightonce
Nematodes	1	Levamisole	Oral	10 mg/ kg bodyweight per day for 7 days
	2	Piperazine sulfate	Oral	10 mg/ kg of body weight per day for 3 days
	2	Fenbendazole	LTB	2 ppm once per week for 3 weeks
			Oral	25mg/ kg body weight per day for 3 days

1.5. Crustacean and annelid parasites of fish

Disease/ Pathogen	Level	Drug/Chemical	Method of administration	Dosage
Argulus *Lernea* *Ergasilus* *Isopod*	1	Ivermectin	Oral	50 g/kg body weight
		$KMnO_4$	LTB	5 ppm for 5-7 days
	2	NaCl	STB	10-20 ppt for 15 minutes
		Delta methrin (Butox)	STB	2- 3 ml/1000 L for 40 minutes
			LTB	35 ml/ha
	2	Dichlorvos	STB	2-3 ppm for 30-60 minutes or 15 ppm for 1 minute
Leech	1	NaCl	STB	2-3%

1.6. Diseases of Shell fish

Disease/ Pathogen	Level	Drug/Chemical	Method of administration	Dosage
Protozoan fouling	1	BKC-50%	LTB	0.5-1 ppm
Bacteria	1	Iodophores (10% iodine)	Dip	20 ppm for surface disinfection of broodstock
Enterocytozoonh epatopenaei	1	CaO	Directly to soil	6 tons per hectare, followed by thorough ploughing and maintaining moist conditions for about a week to raise the soil pH to 12

Note: *1.* $KMnO_4$ *should be used in evening hours/ cloudy days, as it will degrade in sunlight.*

2. Malachite *green should not be used for scale-less fish.*
3. *Formalin and* Malachite *green should not be used in food fish and it should be used for others only with aeration facility.*
4. *Level 1 is preferred method over level 2*

2. USE OF ANTIBIOTIC

- ✓ Never use as a prophylactic agent;
- ✓ Use only when bacterial disease is diagnosed clinically;
- ✓ Use in the recommended dosage and duration;
- ✓ Use appropriate antibiotic based on anti-bio-gram;
- ✓ Avoid repeated use of the same antibiotic;
- ✓ Avoid multiple drugs and indiscriminate use;
- ✓ Commence treatment as early as possible;
- ✓ Avoid oral therapy if fish are off-fed;
- ✓ Adhere to careful storage of antibiotics;
- ✓ Harvesting should be done only after 30 days from the last administration of the antibiotic;
- ✓ The clinical practitioner should record the details such as date of examination, client, diagnosis, product (antibiotic) prescribed, dosage, quantity of fish treated and duration of treatment.

Disease/ Pathogen	Drug/Chemical	Method of administration	Dosage
Pseudomonas (Fin and Tail rot) Aeromonas (Fin and Tail rot, Erythrodermatitis Exophthalmia, Dropsy)	Oxytetracycline	STB	100 ppm for 1 hour in freshwater or 400 ppm for 1 hour in saltwater and repeat for 7 days (for surface bacterial Infections)
		LTB	20- 100 ppm for 3 days. If no recovery, repeat on the third day after 50% water change. Keep the tank covered during treatment to prevent photo-inactivation
		Oral	30 mg/ kg fish twice daily for 5-10 days
		Injection	25- 50 mg/ kg of body weight once (IM or IP)
Streptococcus (Exophthalmia)	Oxytetracycline	Oral	30 mg/ kg fish twice daily for 5-10 days
	Doxycycline*	Injection	2 mg/ kg body weight of fish once
	Sodium Sulfa-mono-methoxine	Injection	50 mg/ kg body weight of fish for 8 days

Flexibacter/ Flavobacteria (Columnaris)	Sulfa-di-methoxine	Oral	10-20 mg/ kg body weight of fish for 3 days
Other Bacteria	Sulfa-di-methoxine	Oral	10-20 mg/ kg body weight of fish for 3 days
	Sodium Sulfa-mono-methoxine + Ormethoprim	Oral	42 mg+ 8mg per kg feed for 3 days
	Sodium Sulfa-mono-methoxine	Bath	28 ppm for 6-10 hours once daily
Hexamita (Hexamitosis - small holes in the head)	Metronidazole	Oral	50 mg/ kg feed once a day for 5 days or 100 mg /kg of body weight for 3 days
	Metronidazole	Bath	5 ppm for 3 hours, repeat every other day for 3 treatments
For Shell fish			
Vibriosis, Black spot disease	Oxytetracycline	Oral	1.5 g/kg feed for 10-14 days

* Should limit the use with ornamental fish

3. MODE OF DRUG ADMINISTRATION

3.1. Water medication

It is the widely practiced mode of drug administration to treat external infection of large numbers of fish. In long term bath treatment, the medicine is added to the water inhabited by the aquatic animals. Here more quantity of medicine is required. In immersion or dipping or short time bathing, small volume of medicated water is prepared in a separate container. The fish, usually held in a net, are immersed in it for a short period of time and then returned to their normal environment.

3.2. Feed medication

It is practiced by oral administration through medicated feed and there is wastage of drug less than water medication. It is adopted for curing internal or systemic diseases. Medicated feed is made by adding the required medication into the feed at the right dose in commercial feed by spraying on the feed using a sprayer and then drying in shade. Alternatively, mix the feed with the correct dose of drug in a suitable binder such as gelatin in a container thoroughly. Add just enough warm water to mix completely. Then dry in shade.

$$\text{\% of drug in the feed} = \frac{0.01\text{x Drug (in mg/kg body weight/day)}}{\text{Feed (\% of body weight/day)}}$$

3.3. Injection

In the advanced stage, intramuscular or intra-peritoneal injection is preferred. Intramuscular injection is given into the epaxial musculature, normally approximately mid-way between the mid-dorsal line and the lateral line. Intra-peritoneal injection is made into the mid-ventral line anterior to the vent, but sometimes causes peritoneal adhesions. The fish should be anaesthetized for giving injection to avoid any injury to be caused to the fish and the operator. Machines can be used for the rapid injection of large numbers of fish. Anaesthesia is not needed for the injection with the machine.

$$\text{Volume of antibiotic} = \frac{\text{recommended dosage (mg/kg)x weight of fish (kg)}}{\text{Solution concentration (mg/ml)}}$$

4. INTENSIVE CRITICAL CARE UNIT

Administration of antibiotics is advisable only in a closed and controlled farming system. If the farming system is extensive, use of antibiotic is not recommended in view of antimicrobial resistance. Moreover, adoption of therapeutic measures under extensive farming system will not be economically viable. In such situation, fish can be shifted to a quarantine pond or tank and kept in 50% new water (preferably chlorinated and dechlorinated). The stocking density can be up to 100 kg/m^3 water with continuous oxygen supply through diffuser.

5. ANESTHETICS

MS 222 at a dose of 15-50 ppm is used as anesthetic before administration of drugs via injection. It is however very expensive. Clove oil (0.05 ml of clove oil per 500 ml of water) can also be used.

6. DRUG METABOLISM AND WITHDRAWAL PERIOD

The withdrawal period is the amount of time required following the administration of the last treatment for measurable residues of the drug or a metabolite to decrease to below the established safe level. This safe level is the MRL, or tolerance. The withdrawal period begins on the day after the last treatment has been administered. The Maximum Residual Limit (MRL) permissible for each antibiotic in aquaculture product as per the guidelines of the Ministry of Commerce and Industries, Government of India is given below as table.

Table: *Commonly used antibiotic and their residual limit*

Antibiotic	Max. Residual Limit (ppm)
Chloramphenicol	Nil
Furazolidone	Nil
Neomycin	Nil
Naldixic acid	Nil
Sulphamethoxazole	Nil
Tetracycline	0.1
Oxtetracycline	0.1
Oxolinic acid	0.3
Trimethoprim	0.05

VIII. PREVENTION OF DISEASES IN AQUACULTURE THROUGH BEST MANAGEMENT PRACTICES (BMP)

Aquatic environment is complex and dynamic in nature. Factors such as presence of pathogen, poor condition of animal and degradation of environment contribute to disease outbreak. Water acts as a good medium for disease. Intensification of aquaculture practices have significantly increased the incidence of disease outbreaks which often leads to huge economic losses. Even though some therapeutics have been advocated for a few diseases, their use is limited to certain operational conditions and/ or some treatments cannot be performed during certain periods, (e.g. just before harvesting period), or in some aquaculture units. (e.g. large ponds/ natural water bodies). Hence, prevention of disease by adopting 'Best Management Practices'(BMP) consisting of proper prophylaxis and bio-security measures is always considered better than treatment. BMP in aquaculture to prevent fish disease includes:

1. DISINFECTION TO CONTROL PATHOGENS DURING PRE-STOCKING:

- ✓ Removal of bottom soil with accumulated organic matter from the earlier crop;
- ✓ Allow the pond bottom to dry and crack to a depth of 25 - 50 mm, to oxidize the remaining organic matter;
- ✓ Disinfection of pond bottom to remove carriers and microbes by sun drying (most of the pathogens, leeches, fish lice, predatory larvae of water insects, eggs and spores of parasites do not survive when relative moisture of soil has dropped to 10 – 15 %)
- ✓ Tilling of pond bottom to enhance drying rate, to accelerate decomposition and mineralization of organic matter and to release noxious gases;
- ✓ Liming of pond to neutralize soil acidity and increase total alkalinity and total hardness;
- ✓ Application of disinfectants, where complete drying is not possible as in case of acid sulphate soil, some of the disinfectants with dosage of application are given below:

Disinfectant	Dose
Bleaching powder	350-500 Kg/ha
Potassium permanganate	5 g/L
Burnt lime (pond bottom)	2.5 – 3 MT/ha
Chloramine –T (chlorseptol)*	30 g/L

*For treatment of concrete structures, troughs and equipment

2. BIOSECURITY MEASURES TO PREVENT ENTRY/ TRANSFER OF PATHOGENS:

- ✓ Prevent the entry of wild aquatic animal (predator/ trash fish) which may act as carriers of pathogen;
- ✓ Prevent piscivorous birds from entering into aquaculture units by placing protective nets;
- ✓ Prevent the entry of crab by placing proper fencing;
- ✓ Prevent the entry of coarse/ aquatic weed into culture units as it may act as source of ecto-parasites;
- ✓ Avoid carrier organisms like snail and copepod (snails can be removed by the application of molluscicides after drying of the bottom and placing net at the inlet);
- ✓ Keep disinfection and drying facilities for nets and other appliances;
- ✓ Use separate utensils/ nets/ implements for individual units;
- ✓ Ensure foot dip and hand wash at the entrance of each unit;
- ✓ Ensure tyre wash facility for the vehicles at the entrance of the farm;
- ✓ Check for clandestine transport;
- ✓ Examine and certify all imported seed/ brooder for the absence of any pathogens;
- ✓ Adopt quarantine measures as per standard procedures for any aquatic animal (native or exotic species) introduced from other countries/ regions;
- ✓ Separate the offspring from brood stock immediately after hatching to prevent horizontal transmission of pathogen;
- ✓ Avoid culture of different age groups in the same unit;
- ✓ Avoid the practice of procuring nauplius/ spawn in seed production facilities from other units in order to prevent dissemination of diseases;
- ✓ Ensure safe and harmless disposal of dead aquatic animals by burning or deep burial (make a pit having a depth of 1.5 - 3 m at least 20 m away from the culture units, layer the bottom of the pit with burnt or chlorinated lime, place the carcass and then cover with at least 80 cm soil.

3. SAFE WATER TO PREVENT THE ENTRY OF PATHOGEN:

- ✓ Use of ground water is ideal as it is generally free from pathogens; however, the sources are limited;
- ✓ Water drawn from open sources must be filtered to reduce the pathogen/ carrier load, use coarse screens to remove larger aquatic animals and debris, followed by progressively finer screens (150–250 µm mesh size) and sand filters (carry out chemical treatment and UV radiation, if required);
- ✓ Ideally should have reservoirs of adequate capacity of culture water for operation;

- ✓ Disinfect the reservoir water to kill any potential vectors or carriers (50 ppm chlorinated water for 24-48 hours) and should be vigorously aerated at least for 48 h to remove residual chlorine (As a commercial bleach powder vary in active chlorine content, dosages need to be adjusted accordingly. Take precautions to protect skin and eyes of personnel);
- ✓ Ensure that water from one culture pond does not enter into reservoir or other culture ponds.

4. SEED AND STOCKING:

- ✓ Record origin, source and health status of spawners and seeds;
- ✓ Use Specific Pathogen Free (SPF) or High Health (HH) broodstock for seed production;
- ✓ Ensure that no banned antibiotic/ disinfectant are used in larval rearing;
- ✓ Check survival rate of larvae within the hatchery and nursery;
- ✓ Ensure seed quality through stress tests;
- ✓ Conduct PCR screening for potential pathogens prior to seed procurement;
- ✓ Ensure minimum transportation stress;
- ✓ Ensure that the stocking density is within the recommended level;
- ✓ Disinfect the seed using 0.5 -1 % common salt (for freshwater fish) or 5 ppm potassium permanganate for 10 minutes under constant observation;
- ✓ Stock the seed only during the early or late hours of the day after proper acclimatization;
- ✓ Ensure sufficient quantity of natural food of appropriate size and species composition before stocking.

5. FEED AND FEEDING:

- ✓ Develop maximum natural food by adequate interventions;
- ✓ Ensure balanced diet (adequate protein, fat, vitamins and mineral);
- ✓ Avoid trash fish as far as possible, since it may act as carrier of pathogens;
- ✓ Ensure antibiotics and pathogen free feed;
- ✓ Monitor feed consumption to avoid over and under feeding;
- ✓ Strictly adhere to recommended feeding frequency and time;
- ✓ Ensure proper feed storage.

6. OPTIMUM ENVIRONMENTAL CONDITION TO REDUCE STRESS:

- ✓ Analyse water quality parameters regularly and maintain at optimum level with minimum fluctuation;

- ✓ Monitor water colour, turbidity, bloom and presence of bioluminescence (during night);
- ✓ Analyse soil quality parameters periodically;
- ✓ Reduce build up of organic load;
- ✓ Prevent inflow of polluted water into farming system;
- ✓ Adopt proper water treatment methods;
- ✓ Ensure placement of water inlet and outlet at diagonally opposite positions or as distant as possible;
- ✓ Use zeolite or appropriate bio-remediators/ probiotics to ensure optimal water quality;
- ✓ Use mechanical aerators;
- ✓ In cage farming, ensure proper water flow by regular cleaning/ changing net;
- ✓ Adhere to recommended carrying capacity through cull harvesting.

7. HEALTH MONITORING

- ✓ Monitor the growth and health by random sampling through cast netting and feed trays, once in a week for intensive system and once in a month for extensive system (avoid sampling during the first month);
- ✓ Adopt prophylactic measures by the application of prebiotics, probiotics, nutraceuticals and immunostimulants;
- ✓ Eliminate pathogens and their carriers effectively;
- ✓ Ensure the animal is free from chemicals or unapproved drugs;
- ✓ Identify and minimise the potential risk factors to health throughout the culture.

8. DISINFECTION OF EFFLUENT:

- ✓ Treat the effluent with bleaching powder in effluent tank to maintain 50 ppm available chlorine for 24-48 hours;
- ✓ Vigorously aerate the chlorinated effluent at least for 48 hours to remove residual chlorine.

9. RECORD KEEPING

- ✓ Maintain farm health card for prompt, accurate and complete record keeping about the stock (it is imperative in good health management to detect trends before problems become intractable and to initiate actions most effectively) and it should contain the following:-
  - Details of seed (species, source and screening details);
  - Details of stocking (date and density);
  - Size of animal (length or weight);
  - Soil and water quality data;
  - Details of feed (source & batch, type, proximate composition, feeding rate & regime, quality check and consumption rate);
  - Time and rate of water exchange;
  - Data on growth and survival;
  - Reports of disease incidence, investigation, treatment and control measures;
  - Details of prophylactics (chemicals, immunostimulants or bioremediators).

10. DISEASE SURVEILLANCE

- ✓ Familiarise the biology of host/ animal, its environment and etiology of diseases;
- ✓ Eliminate or restrict sources and the possibilities of infection (invasion) and its further expansion;
- ✓ Improve the health status of animal to withstand the infection (invasion);
- ✓ Follow effective quarantine guidelines and health certification procedures to minimize the risk of introduction and spread of pathogens;
- ✓ Develop scientific and functional disease reporting system from farm level onwards;
- ✓ Establish health information system at district and state level through farmer level linkage;
- ✓ Develop an early warning/ forecasting system to manage outbreak of diseases;
- ✓ Establish effective extension system through improved infrastructure and trained/ qualified human resource.

11. DISINFECTION OF INFECTED FARMING SYSTEM:

- ✓ Do not discharge water from infected ponds without treatment;
- ✓ Remove aeration devices and implements and disinfect separately;
- ✓ Disinfect by evenly distributing calcium hypochlorite to provide a minimum final free chlorine concentration of 50 ppm in the water and allow it to stand for a minimum

of 24–48 hours at this minimal chlorine concentration by adding more calcium hypochlorite, if required;

- ✓ Remove deposits of organic debris in the pond bottom;
- ✓ Sun dry for at least three weeks;
- ✓ Ensure that there are no wet patches in the ponds at the centre or near the sluice gates;
- ✓ Plough, till and level the pond;
- ✓ After disinfection, treat the dried earthen pond with quicklime (calcium oxide) at the rate of 4–6 MT/ ha for desiccation/ dehydration of organic matter.

IX. AQUATIC QUARANTINE PRACTICES

Active transport of live aquatic animals including import for aquaculture may lead to the introduction of known, emerging or re-emerging pathogens inducing diseases that may result in socio-economic and ecological damages. Hence, proper quarantine practices have to be undertaken while procuring new stock or importing aquatic animals. Quarantine is the maintenance of aquatic animals in isolation under observation for specific duration with requisite tests and treatment. Based on clinical analysis, decision should be made to release or destroy the stock.

The quarantine facility should have adequate infrastructure supported with adequate water, uninterrupted power and related life support system, operating protocols and well-trained and dedicated human resources. It should not be installed in the vicinity of any aquaculture facilities, water sources or areas subject to frequent flooding. The facility should not be used for any purpose, other than quarantine. Increased stress to the quarantined animals may help in the overt expression of subclinical infections which otherwise go unnoticed. Eggs, embryonic or larval stages carry fewer subclinical infections compared to adult and are considered low risk and hence easy to quarantine.

1. COMPONENTS OF AQUATIC QUARANTINE FACILITY (AQF)

1.1. Bio-security:

- ✓ Ensure a foolproof boundary with compound wall around the premises, with adequate height to prevent the entry of animals and unauthorized personnel;
- ✓ Display signs prominently on all sides of the external fencing and on all entrances to the facility to show that it is an Aquatic Quarantine Facility (AQF) and unauthorized entry is prohibited;
- ✓ Provide tyre dip at the gate of entry for disinfection of vehicle;
- ✓ Lock the AQF and its fencing securely when it is not in active use;
- ✓ Ensure separate change room for showering and changing outer clothing prior to entry/ exit.

1.2. Receiving area:

- ✓ Earmark a designated area for receiving the animal into the AQF.

1.3. Quarantine cubicle:

- ✓ Place footbath containing disinfectant at the entrance door;
- ✓ Provide facility for hand disinfection at entry and exit of the unit;

- ✓ Ensure that floor is constructed of concrete and cladded with tiles or other impervious material with sufficient slope to drain into an enclosed holding tank for disinfection;
- ✓ Install floor drainage with control mechanism to prevent the accidental escape of aquatic animals or uncontrolled release of water;
- ✓ Ensure that walls be cladded with tiles or other impervious material up to a minimum height of 180 cm;
- ✓ Ensure that floor to wall junction and any gap or crack in wall, floor or ceiling should be effectively sealed for containing any leaks;
- ✓ Equip waterproof half glassed doors with self-closing mechanisms;
- ✓ Screen any openings to prevent the entry of birds/ insects;
- ✓ Provide enough holding capacity for the anticipated quantity of imports;
- ✓ Ensure separate cubicles in quarantine area for different importers;
- ✓ Ensure that each cubicle for ornamental fish should be about 200 square feet area and may hold about 20 aquaria of 50-100 L capacities with about 1-2 kg total biomass and that of food fish should be about 300 square feet area with sufficient quarantine tanks of 500 L to hold 15 kg biomass;
- ✓ Each cubicle shall be air conditioned to maintain a temperature of 25^0 C;
- ✓ Provide adequate lighting to allow proper inspection of all animals;
- ✓ Equip with back-up systems for essential components (e.g. electricity, water, aeration, temperature control and filtration) in case of electrical or mechanical failure;
- ✓ Ensure separate provision for stress lab as the stress condition favors the onset of clinical sign or symptoms of disease. (If the animal is in comfort zone the subclinical form of disease may not be visible during quarantine for 7 days and stress test can be opted depending on the number and cost of animal imported).

1.4. Quarantine tank:

- ✓ Provide a permanent number to each individual tank;
- ✓ Provide lid to prevent transmission of pathogens between adjacent tanks due to splash from the aeration/ filter system/ aerosols, and escape of aquatic animal;
- ✓ Arrange tanks in rows with minimum gap of 75 cm between rows of tanks or tanks and walls for inspection purposes;
- ✓ Ensure that at least the front portion of each tank is transparent to provide good visibility of the contents;
- ✓ Equip water intake lines with automatic shut-off valves.

1.5. Fish feed storage:

- ✓ Provide secured, cool and dry facility for feed storage to prevent any contamination or infestation.

1.6. Diagnostic laboratory:

- ✓ Ensure microbiology, histopathology and PCR laboratory facilities;
- ✓ Personnel employed should have sound knowledge and exposure in parasitology, bacteriology, mycology, virology and histopathology of aquatic animals.

1.7. Packing and delivery section:

- ✓ Ensure separate packing and delivery sections.

1.8. Water supply:

- ✓ Ensure that groundwater source is free from contaminants including any possible infective agents;
- ✓ Ensure water supply system with adequate treatment and disinfection (mesh bags, sand filters, ozonation and UV treatment) to avoid vectors and any pathogens;
- ✓ Provide independent bio-filtration system for each tank.

1.9. Blower room:

- ✓ Ascertain that all air compressors used for aquaculture purposes are "oil-less", with an additional one for emergency backup.

1.10. Freezer:

- ✓ Provide with a designated freezer (preferably -80^{0}C) within the quarantine area solely for the storage and preservation of dead aquatic animals.

1.11. Provision for disinfecting equipment:

- ✓ Ensure a suitable wash-up trough in the quarantine area for the cleaning and disinfection of implements;
- ✓ Provide a suitable draining rack for air drying of implements.

1.12. Effluent Treatment System (ETS) :

- ✓ Ensure well designed ETS to handle the disinfection of both the overseas transport water and domestic water used in the AQF.

1.13. Incinerator:

- ✓ Install an appropriate incinerator in the quarantine for disposal of carcasses and infected stock.

1.14. Amenities for Quarantine Personnel:

- ✓ Provide ample amenities and safety precautions for quarantine personnel;
- ✓ Provide and maintain a fully stocked first aid cabinet.

2. PROCESS OF AQUATIC QUARANTINE OPERATION

2.1. Clearance at port of entry:

- ✓ Receive the consignment after clearance from port of entry by the quarantine staff (with dress code and ID cards/ badges);
- ✓ Observe the condition and number of cartons and record damages if any in presence of the importer;
- ✓ Transport the consignment to the quarantine facility in the quarantine vehicle in pre-designated route without delay.

2.2. Receiving at the AQF:

- ✓ Ensure tyre bath of the vehicle while entering;
- ✓ Unload the entire consignment at the receiving area (all the staff involved in unloading must disinfect their hands, apron, coat and shoes)
- ✓ Permit one representative of the importer/ consignee to observe the animals in the receiving area;
- ✓ Disinfect or incinerate all containers (bags, boxes and cartons) used to hold aquatic animals during transit under the supervision of the quarantine staff. Keep a record of all dead/ moribund animals.

2.3. Pre-Quarantine operations:

- ✓ Shift the polythene bag with animal only for pre-quarantine;
- ✓ Keep the polythene bags with fish in the tank with filtered, chlorinated, de-chlorinated, UV sterilized water for acclimatization;
- ✓ Remove the dead/ moribund animal immediately for proper clinical examination and disposal by sterilization using an autoclave, followed by incineration or deep burial;
- ✓ Assess and acknowledge the general health condition of the animal jointly by AQF technical staff and the importer in the prescribed format;
- ✓ Report immediately to the supervising Quarantine Officer about unusual levels of mortality, changes in behavior or unusual signs of disease, parasites or pests, if any;
- ✓ Transfer the animal to the quarantine unit.

2.4. Testing for pathogens

- ✓ Conduct clinical examination of the aquatic animal for external parasites;
- ✓ Take samples of skin scrape, fin clip and gill clip and examine under a microscope;

- ✓ Take faecal samples from the polythene bag (may be pooled) and tested in the laboratory as per OIE protocol for the concerned pathogens;
- ✓ Perform necropsy including microbiology to rule out the presence of bacterial, viral and fungal diseases, as well as to identify internal parasites, if there is moribund specimen;
- ✓ Send stored samples immediately to accredited research institutions for further confirmation, in case any of the samples test positive;
- ✓ Report to the State Level Surveillance Committee for aquatic animal disease; if confirmed, the final decision on destroying the entire consignment to be taken within 24 hours and intimated to the importer;
- ✓ Destroy the entire consignment at importer's cost as per the prescribed protocols, if the consignment does not pass quality test;
- ✓ Destroy the entire stock once a serious untreatable disease or pathogen is encountered in aquatic animals held in quarantine and disinfect the facility appropriately;
- ✓ Notify the presence of disease in the consignment to the source company by the quarantine officer (If two consignments are tested positive for any OIE listed diseases, the supplier shall be recommended for blacklisting).

2.5. Quarantine operations:

- ✓ Restrict each cubicle for a single batch;
- ✓ Avoid mixing of animals, water or equipment between the tanks;
- ✓ Ensure that clearly marked and separate set of net, bucket, beaker and other implements be placed in a cubicle, to avoid sharing between batches;
- ✓ Use sterilizable/ disposable materials for any operation;
- ✓ Feed the animal with bio-secure feed based on demand (Consumption shall be checked in each tank one hour after feeding and recorded. The ration shall be appropriately increased/ decreased based on the level of consumption);
- ✓ Siphon out unfed feed and faeces twice daily from the holding tank;
- ✓ Replenish lost water to maintain the water level;
- ✓ Store the feed in separate containers hygienically under refrigeration;
- ✓ Analyze water quality for pH and ammonia daily for each tank;
- ✓ Restrict the entry into the rooms except for feeding, water exchange and monitoring;
- ✓ Conduct quarantine operations for 7-14 days for each batch;
- ✓ Wash and disinfect used plastic containers and hoses with hypochlorite solution (100 ppm) before reuse.

2.6. Dispatch of certified animal

- ✓ Dip in 200 ppm formalin for 2-3 minutes followed by a rinse in fresh water;
- ✓ Pack in polythene bags with oxygen at the packing area and hand over to the importer, in case the animals are found to be negative for tested pathogens;
- ✓ Provide the importer with delivery note along with Quarantine Certificate (QC) by the Quarantine Officer;
- ✓ Clean and disinfect the utilized area thoroughly after each cycle of operation.

2.7. Disinfection of equipment:

- ✓ Clean and disinfect all tanks and equipment thoroughly with hypochlorite solution at 200 ppm available chlorine for 12 hours before restocking.

2.8. Effluent disposal:

- ✓ Avoid discharge of the wastewater directly into natural waterways;
- ✓ Conform to state/ local government regulations for disposal of wastewater;
- ✓ Disinfect the discharged wastewater from the quarantine facility with chlorination (It is particularly crucial where the water is to be discharged to the same location as the abstraction point) and in accordance with following steps:
  - o Should pass through an approved filter capable of removing suspended organic material prior to hypochlorite treatment.
  - o Should pass to a retention vessel where sufficient hypochlorite is to be added to achieve a minimum concentration of 200 ppm at 12 hours post-treatment. Sodium hypochlorite can be used at 1.6 ml of hypochlorite solution (12.5% available chlorine) per litre of water, while calcium hypochlorite powder (65-70% available chlorine) should be used at 0.3 g of powder per litre of water.
  - o Measure the chlorine concentration after a retention period of 3 hours and re-treat if necessary to maintain the concentration;
  - o Do not discharge chlorinated water directly into adjacent waterways (Neutralize the chlorine in the wastewater by adding sodium thiosulphate at a rate of 1.25 g per litre of treated wastewater and by agitating for not less than 10 min before discharge).
  - o Maintain chlorination records with details noting on the amount of compound added, the volume of effluent, the time at which treatment period commenced, the pH at commencement of the treatment period, the 12 hours. Post treatment concentration, the amount of sodium thiosulphate added to achieve neutralization and the residual chlorine concentration at discharge.

2.9. Operation Management:

- ✓ Ensure that the operations of AQF are under strict bio-security protocol;
- ✓ Develop a contingency plan addressing actions to be taken in the event of a vehicle breakdown during transport of aquatic animals from customs arrival to the Quarantine facility, and during on-site emergencies that may arise due to improper handling or natural calamities such as fire, flood, lightning etc;
- ✓ Undertake systematic periodic internal audits at least on a quarterly basis to ensure standards of operation and to identify and correct deficiencies if any;
- ✓ Develop detailed manual on the operation of different sections such as bio-security protocol, diagnostic tests, water treatment, water quality management, feeding, staff personal hygiene and other management protocols.

2.10. Personnel hygiene

- ✓ Entry and exit to the quarantine unit should be after shower and change into personal protective clothes and boots;
- ✓ Restrict the entry to the minimum required to perform necessary maintenance and observation of the quarantined animal;
- ✓ Do not allow food stuff in the quarantine zone;
- ✓ Record the movement of personnel in the facility-electronically and also by the security personnel.

2.11. Record keeping

- ✓ Maintain data sheets on the activities during the period of holding at AQF which includes the details of diagnosis, water quality characteristics, mortality etc;
- ✓ Maintain a complete history of the stock of animals being contained in the AQF;
- ✓ Maintain all documentation (copies of shipping bills, health certificates, bio-security clearance, etc.) and records for a minimum period of 36 months for auditing purposes;
- ✓ Maintain record sheet for each tank up to date and it must consist of:

    - Number of aquatic animal in tank;
    - Details of exporter including country of export;
    - Name of importer, shipment or airway bill number and date of arrival;
    - Number of aquatic animal found dead on arrival;
    - Details of any observed disease conditions and number of sick aquatic animals;

  - Daily record of number of aquatic animal deaths in tank;
  - Details of disposal and disinfection;
  - Details of any progeny produced (date and number) and the corresponding number of the tank to which it is transferred.

2.12. Online documentation:

- ✓ Enable an online system to reserve the quarantine space as per requirement and also keep the importer informed about every details with utmost transparency;
- ✓ Provide online access for the Quarantine Committee Members to the details regarding cubicle booking, import permits, utilization, cancellation, import and dispatch of fish;
- ✓ Provide database on list of permitted exotic species and list of diseases of national concern.

ANNEXURE-1

COMPOSITION OF FIXATIVES

Neutral Buffered Formalin (NBF)

Formalin	:100 ml
Tap Water	: 900 ml
Sodium dihydrogen phosphate, monohydrate	: 4.0 g
Disodium hydrogen Phosphate, anhydrous (Na_2HPO_4)	: 6.5 g

Davidson's AFA (alcohol, formalin, acetic acid) fixative for shrimps

Ethyl alcohol (95%)	: 330 ml
Formalin	: 220 ml
Glacial acetic acid	: 115 ml
Tap water	: 335 ml

(Store the fixative in glass or plastic bottles with secure caps at room temperature)

ANNEXURE-2
COMPOSITION OF MEDIA

(Weight of each ingredient is given in gm/lit)

Zobell Marine Agar (ZMA) pH -7.6±0.2

Peptic digest of animal tissue	5.00
Yeast extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Calcium chloride	1.80
Potassium chloride	0.55
Sodium bicarbonate	0.16
Sodium sulphate	3.24
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.00

Brain Heart Infusion Agar (BHIA) pH -7.4±0.2

Calf brain, infusion from	12.50
Beef heart, infusion from	5.00
Peptic Digest	10.00
Dextrose	2.00
Sodium chloride	5.00
Disodium phosphate	2.50
Agar	15.00

TCBS Agar - pH -8.6±0.2

Proteose peptone	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Oxgall	10.00
Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.00

Tryptose yeast extract salt Agar

$MgSo_4$	0.5 g
$CaCl_2$	0.5 g
Yeast extract	0.4 g
Tryptone	4.0 g
Agar	1.5
Dw	make upto 1000 ml

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